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Members of the erbB family of receptor tyrosine kinases include the epidermal growth factor (EGF) receptor and erbB2 (also known as HER-2/Neu) that is found overexpressed in many human breast cancer cases. The aim of our studies is to understand the mechanism by which growth factors activate these receptors. If this mechanism can be understood in detail, it should be possible to design approaches to block inappropriate receptor activation, which occurs in many breast cancers. While EGF activates erbB1 (the EGF receptor) by directly inducing its homodimerization, the same growth factor activates erbB2 by inducing the formation of hetero-oligomeric complexes between erbB1 and erbB2. We have shown that the isolated extracellular domain of erbB1 is sufficient for EGF-induced homodimerization of that receptor. By contrast, isolated extracellular domains are not sufficient to recapitulate the hetero-oligomerization of erbB receptors that has been observed *in vivo*. These findings argue that erbB receptor homo- and hetero-oligomerization occur though different mechanisms. Subsequently, using a variety of approaches for analyzing erbB receptor transmodulation in living cells, we have generated data that support a homodimer-nucleated heterotetramer model. In this case, an activated erbB1 homodimer is the effective ligand for erbB2, and activates it. This view, although preliminary, provides new ideas for developing approaches to reverse aberrant erbB2 activation in breast cancer.

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TABLE OF CONTENTS

INTRODUCTION

Cellular responses to many extracellular factors that control cell growth and differentiation are mediated by cell-surface receptors with tyrosine kinase activity. The mechanism of transmembrane signaling by receptor tyrosine kinases involves an initial ligand-induced receptor dimerization event that leads to activation of the intracellular tyrosine kinase domain. For a given class of receptor tyrosine kinases, ligand-induced dimerization can involve two receptors that are the same (homodimerization) or different (heterodimerization). It is now appreciated that heterodimerization provides a mechanism for increasing the diversity of signaling through a given family of receptors. In our studies we focus on the four known receptors in the epidermal growth factor (EGF) receptor family - known as erbB1 to erbB4. The erbB receptors have been implicated in a number of human cancers. In particular, erbB2 (also known as Neu, or HER-2) is strongly implicated in breast cancer. Aberrant overexpression of a single member of this family can disrupt normal signaling, in some cases leading to uncontrolled cell proliferation. There are at least 12 different ligands that signal through the erbB family of receptor tyrosine kinases, including EGF, TGFα, and the neuregulins. The ligands differ in their receptor-binding characteristics, and appear to induce formation of distinct combinations of erbB receptor homo- and heterodimers. Their specific biological activities are thought to arise from these differences. We are interested in understanding how the multiple different ligands induce formation of particular receptor dimers. For the EGF receptor (erbB1), we previously showed that the extracellular domain is sufficient for ligand-induced dimerization, and that two EGF molecules are required to form the dimer. Through biophysical analyses of the other erbB receptor extracellular domains, produced in a baculovirus expression system, we have compared ligand-induced receptor homo- and heterodimerization by EGF and neuregulin-1β (NRG1-β), the results of which are described in this report. Our current findings indicate that hetero-oligomerization or transmodulation of erbB receptors differs mechanistically from the accepted ligand-induced homodimerization model established for these and other receptors. Furthermore, while ligand-induced heterodimerization may be relevant for erbB2/erbB3/erbB4, it appears not to be important for the EGF receptor (erbB1). Our next goal is to incorporate the results of these studies of dimerization *in vitro* into an *in vivo* picture of signaling by this class of receptors. By developing this understanding, we hope that approaches will be suggested for specifically modulating erbB receptor signaling when it is disrupted in human cancers.

BODY OF PROGRESS REPORT

Progress for each task in the Statement of Work is detailed following description of the task.

Task 1.

To determine quantitatively the hierarchy of erbB receptor homo- and heterodimers induced by each ligand in the EGF and NRG family

- *generate s-erbB proteins and erbB ligand in insect cells and yeast/bacteria, and perform preliminary qualitative studies of ligand-induced homo- and heterodimerization (months -18 to 0)*

We succeeded in producing milligram quantities of the four erbB receptor extracellular domains before funding of the award. Each of the four erbB receptor extracellular domains (s-erbB1, s-erbB2, s-erbB3, and s-erbB4) was secreted from Sf9 cells infected with recombinant baculovirus. Each s-erbB protein included the entire extracellular domain of the relevant receptor, followed by a hexahistidine tag to expedite purification from conditioned insect-cell medium. The most C-terminal native amino acid of each protein was K642 (s-erbB1); P647 (s-erbB2); K639 (s-erbB3); and R649 (s-erbB4); where residue numbers include the signal peptide.

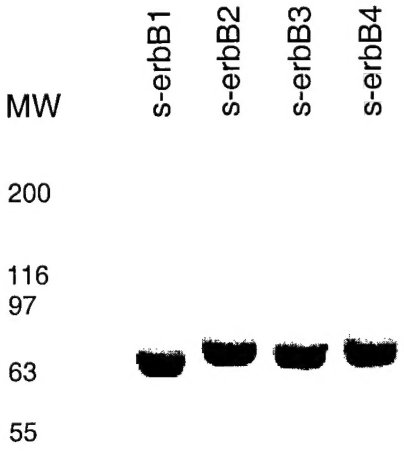


Figure 1

SDS-PAGE of the purified s-erbB proteins used for biophysical analyses of ligand-induced homo- and hetero-dimerization described in this progress report.

Using a Ni-NTA agarose column, followed by gel filtration and a single round of ion exchange (see Experimental Procedures), s-erbB proteins could be prepared from Sf9-cell conditioned, serum-free, medium with yields of 1.5 mg/liter (s-erbB1), 0.3 mg/liter (s-erbB2), 1 mg/liter (s-erbB3), and 0.8 mg/liter (s-erbB4). Significantly higher total yields could be achieved using High Five cells from *T. ni*, but the purified protein from these cells was more heterogeneous, and in some cases showed a tendency to aggregate. As a consequence, only protein secreted by Sf9 cells was employed in the studies described here. Figure 1 shows a Coomassie-stained SDS-PAGE gel of the purified s-erbB proteins, which we estimate to be greater than 92-95% pure in all cases. In more recent studies (2001) we have begun to employ a Schneider 2 (S2) *Drosophila* cell expression system, and yields appear to be improved.

We have focused most of our studies to date on the central ligands of the erbB ligand family: epidermal growth factor (EGF) and neuregulin-1β1 (NRG1-β1). A number of experiments have also been performed with heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor-

α (TGF-α). In all cases, commercially available ligands (from Intergen or R & D Systems) have been employed. Our own efforts to express large quantities of erbB ligands in *Pichia pastoris* and *E. coli* yielded good quantities of EGF, which we have used for some experiments. However, NRG's, betacellulin, and TGF-α produced in these systems, while bio-active, were insufficiently homogeneous for use in our biophysical studies. Subsequent efforts allowed us to generate neuregulins 1, 2, 3, and 4 using a Drosophila S2 cell expression system.

- *perform binding assays for each ligand to each possible combination of s-erbB proteins using calorimetric and SPR approaches (months 1 to 18)*

We have used surface plasmon resonance (SPR/BIAcore) to confirm that the s-erbB proteins secreted from Sf9 cells bind to the relevant growth factor ligands. BIAcore CM-5 sensor chips were derivatized with the EGF-like domains of EGF, HB-EGF, NRG1-β1, NRG2-β or with no ligand, and solutions of the four purified s-erbB proteins were passed over the resulting surfaces. The results of these BIAcore studies are summarized in Figure 2 and Table I. As anticipated, s-erbB1 bound significantly to the EGF-derivatized and HB-EGF-derivatized surfaces, but not to surfaces carrying NRG1, NRG2, or no ligand. By contrast, s-erbB2 did not bind to any of the surfaces. Significant binding of s-erbB3 was seen to NRG1-β1 only (not to NRG2), while s-erbB4 bound strongly to NRG1-β1 and NRG2-β, and weakly (but significantly) to HB-EGF, suggesting that this is indeed to some extent a bispecific ligand. Estimated K_D values (Table I) all lie well within the range reported (100 to 500 nM) for EGF binding by monomeric s-erbB1 produced in mammalian cells (Greenfield *et al.*, 1989; Günther *et al.*, 1990; Hurwitz *et al.*, 1991; Lax *et al.*, 1991a; Zhou *et al.*, 1993; Brown *et al.*, 1994; Lemmon *et al.*, 1997).

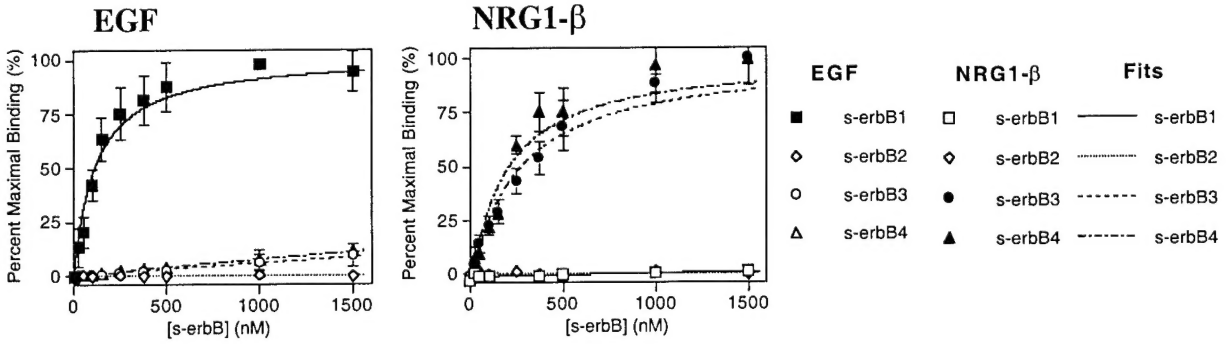


Figure 2
Binding of s-erbB1, s-erbB2, s-erbB3, and s-erbB4 to EGF (left) and NRG1-β1 (right), immobilized on a BIAcore chip. Best-fits to the data, assuming a simple association model, are shown. Errors are standard deviations from the mean of at least 4 independent determinations at each point. K_D values represented by the best fits are list in Table I.

The relative binding affinities listed in Table I agree very well with the relative K_D or IC_{50} values reported from studies of IgG fusion proteins for EGF binding to s-erbB1 compared with binding of NRG1-β to erbB3 and erbB4. A K_D value of 17-35 nM was previously reported for binding of full-length NRG1-β2 to monomeric s-erbB3 from analytical ultracentrifugation studies (Horan *et al.*, 1995). The ≈10-fold higher affinity seen by Horan *et al.*, may result from their use of full-length NRG1-β2 rather than the EGF-like domain alone. However, the EGF-like domain is known to be sufficient for all known biological activities of NRG1 (Holmes *et al.*, 1992). When binding of growth factors to predimerized IgG

fusion proteins of erbB receptor extracellular domains is measured, the apparent affinities are approximately 30-fold higher (Jones *et al.*, 1998, 1999; Ballinger *et al.*, 1998; Fitzpatrick *et al.*, 1998).

Table I

Ligand binding by the s-erbB proteins

Ligand	K_D for s-erbB1 (nM)	K_D for s-erbB2 (nM)	K_D for s-erbB3 (nM)	K_D for s-erbB4 (nM)
EGF	**118 ± 41**	none	$> 10^4$	$> 10^4$
NRG1-β1	$> 10^5$	none	**249 ± 80**	**179 ± 10**
NRG2-β	$> 10^5$	none	$> 10^5$	**500**
HB-EGF	**410**	none	$> 10^5$	$\approx$ **3 x 10^4**

Summary of K_D measurements made for the four s-erbB proteins to immobilized EGF, NRG1-β1, NRG2-β, and HB-EGF. K_D values listed explicitly represent means of at least four independent determinations when quoted alongside their standard deviations.

We have not been able to detect any significant difference in binding affinities when passing over mixtures of s-erbB proteins. In particular, a mixture of s-erbB2 and s-erbB4 gave results for NRG1-β binding that were not clearly distinguishable from those determined with s-erbB4 alone (Figure 2), despite the fact that NRG1-β induces s-erbB2/s-erbB4 heterodimer formation. Titration calorimetry was employed to study EGF binding to s-erbB1, and results identical to those obtained previously (Lemmon et al., 1997) were obtained. Given the relative difficulty of interpreting calorimetric results for dimerization-coupled binding events, and the large quantities of material required for these studies, we have settled upon BIAcore measurements as a more expeditious approach.

- *using classical multi-angle laser light scattering methods, measure the ability of each erbB ligand to induce homodimerization of each s-erbB protein, by varying both ligand and receptor concentration (months 1 to 4)*

As shown in Figure 3, multi-angle laser light-scattering (MALLS) was measured for a series of samples containing s-erbB protein at 4 μM, to which had been added increasing concentrations of EGF or NRG1-β1. The weight-averaged molecular mass ($M_{\bar{w}}$) for each sample, relative to that measured in the absence of ligand, was determined by extrapolation of a Debye plot to zero angle and was expressed as a fold-increase in $M_{\bar{w}}$ (see Experimental Procedures). As shown in Figure 3, addition of one molar equivalent of EGF to s-erbB1 resulted in a doubling of $M_{\bar{w}}$, as we have observed previously in X-ray scattering studies (Lemmon *et al.*, 1997). No further increase in $M_{\bar{w}}$ was seen when larger excesses of EGF were added, consistent with our previous finding that EGF induces formation of a 2:2 s-erbB1:EGF dimer, but no higher order oligomers (Lemmon *et al.*, 1997).

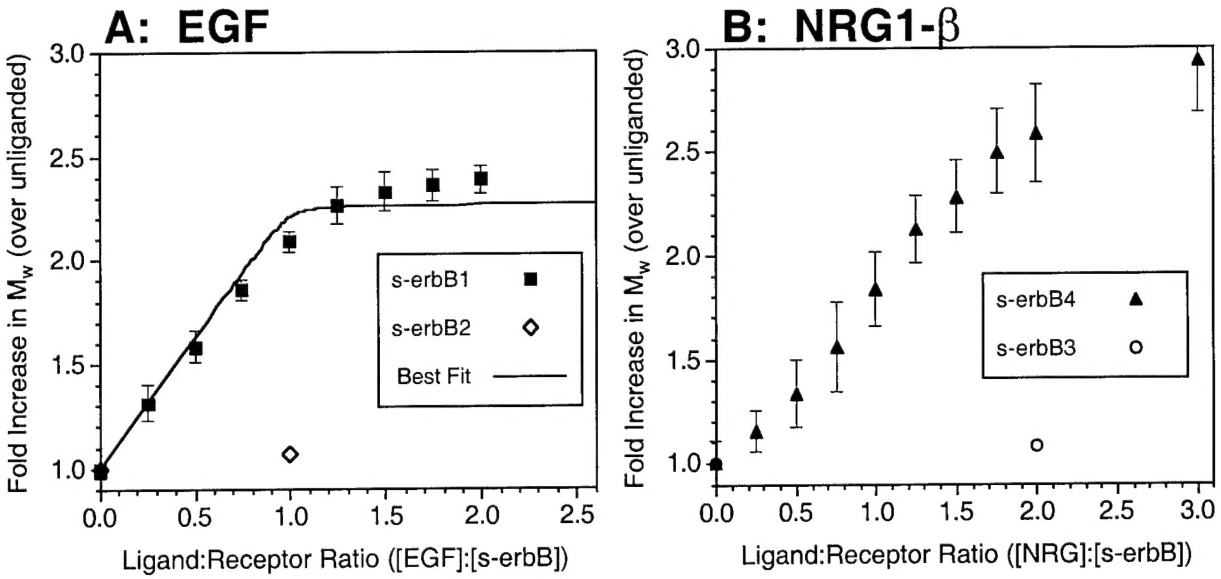


Figure 3
MALLS studies of EGF-induced homo-dimerization of s-erbB1 and s-erbB2 (A) as well as of NRG1-β1 induced homodimerization of s-erbB3 and s-erbB4 (B) (see text).

Figure 3A shows a best fit to the EGF-induced s-erbB1 dimerization data using a model in which a 1:1 EGF:s-erbB1 complex forms with K_D = 118 nM, and this 1:1 complex dimerizes with K_D less than 100 nM to form the 2:2 EGF:s-erbB1 dimer. This data fitting indicates that our insect cell-derived s-erbB1 dimerizes 20 to 50-fold more strongly than the CHO cell-derived protein studied by Lemmon *et al.*

Analysis of s-erbB4 by MALLS also demonstrated clear dimerization upon addition of NRG1-β1. In this case, the maximum $M_{\overline{w}}$ is not reached until almost two equivalents of NRG1-β1 have been added to the s-erbB4. Furthermore, in some experiments the final $M_{\overline{w}}$ was a little higher than expected for a 2:2 s-erbB4:NRG1-β1 complex. These MALLS experiments, together with our analytical ultracentrifugation studies, argue against the possibility that NRG1-β1 induces formation of s-erbB4 oligomers with order greater than two. However, the data are consistent with a model similar to that described for EGF-induced s-erbB1 dimerization if it is assumed that 20 to 30% of the NRG1-β1 preparation is present as small aggregates and/or is inactive. Given this caveat, we have not attempted to fit the NRG1-β1/s-erbB4 data explicitly, although it is clear that NRG1-β1 induces strong s-erbB4 dimerization.

As also shown in Figure 3, addition of excess EGF to s-erbB2 does not induce its dimerization (Fig 3A), and addition of excess NRG1-β1 to s-erbB3 does not significantly increase $M_{\overline{w}}$ of that protein (Fig 3B). Thus, these data argue that EGF induces homodimerization of only s-erbB1, and NRG1-β1 induces homodimerization of only s-erbB4. Homodimers of neither s-erbB2 nor s-erbB3 can be induced by these ligands or any other tested.

To determine K_D values for ligand-induced s-erbB1 and s-erbB4 homodimerization, experiments were performed in which the concentration of a 1:1 receptor:ligand complex was varied, as shown in Figure 4. For the CHO cell-derived s-erbB1 that we employed prior to the beginning of these studies, this approach was useful, and gave a dimerization K_D of approximately 3 μM to 8 μM, depending on the batch of protein employed. The protein that we now produce from insect cells dimerizes much more strongly, such that we have not been able to detect dissociation of the dimer in complex dilution experiments of this sort. Data fitting suggests that K_D for s-erbB1 or s-erbB4 homodimerization, when occupied by EGF or NRG1-β1 respectively, is approximately 30 nM.

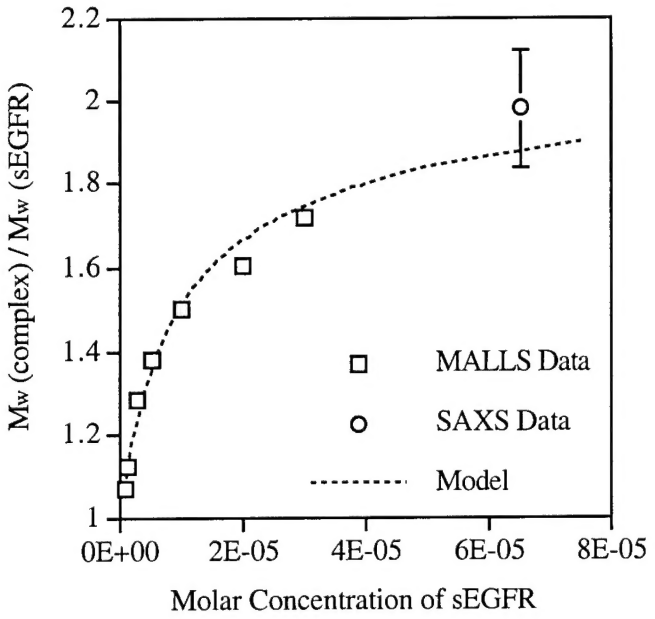


Figure 4
Measurement of $\overline{M}_w$ for a 1:1 EGF/s-erbB1 mixture at a series of different molar concentrations in 50 mM Hepes, pH 7.5, 100 mM NaCl at 25°C. Data up to 30μM were obtained from MALLS measurements, while the data point at 65μM is an average of the relative $\overline{M}_w$ measured for a 1:1 mixture in small-angle X-ray scattering (SAXS) experiments. The solid line represents predicted data from the model presented by Lemmon *et al.*, (1997), with K_γ increased to 8μM and K_β reduced to 25μM as described in the text. The error on the Debye plot fit for each MALLS data point does not extend beyond the symbol size.

- *using the results from the homodimerization assay, assess using light-scattering the ability of each erbB ligand to induce each of the 6 possible s-erbB heterodimers. By repeating experiments using different concentrations of receptors and ligands (and different ratios of the two s-erbB proteins), determine binding constants for dimerization and ligand binding (months 1 to 12)*

The most likely relevant erbB receptor heterodimer or oligomer in breast cancer, and the one that was first reported to occur, is the erbB1/erbB2, or EGFR/Neu hetero-oligomer (King *et al.*, 1988; Stern *et al.*, 1988; Wada *et al.*, 1990). Having established (as described above) that EGF induces efficient s-erbB1 homodimerization, and that NRG1-β1 induces efficient s-erbB4 homodimerization, we next tested the ability of these s-erbB proteins to heterodimerize upon growth factor binding. As outlined in the introduction, there is a great deal of evidence for ligand-induced heterodimerization of erbB receptors. One of the first indications for heterodimerization (or transmodulation) came from the finding that erbB2, which does not bind EGF, can nonetheless be activated by EGF in cells that express both erbB1 and erbB2 (King *et al.*, 1988; Stern *et al.*, 1988; Wada *et al.*, 1990; Spivak-Kroizman *et al.*, 1992). ErbB2 is not activated by EGF in cells that do not express erbB1. Transmodulation of erbB2 by erbB1 has been shown to result from EGF-dependent association of erbB1 and erbB2 to form presumed heterodimers that show elevated tyrosine kinase activity, and are extensively autophosphorylated (Wada *et al.*, 1990; Spivak-Kroizman *et al.*, 1992). Supporting the suggestion that erbB1/erbB2 heterodimers might resemble active erbB1 homodimers, erbB2 with a cytoplasmic truncation was reported to act as a dominant-negative inhibitor of erbB1 signaling (Qian *et al.*, 1994) apparently in the same way as similar erbB1 truncation mutants inhibit EGF signaling (Kashles et al., 1991).

Since EGF efficiently induces s-erbB1 homodimerization (see above), we reasoned from findings in the literature and current models for heterodimerization that EGF should also induce efficient heterodimerization of s-erbB1 and s-erbB2.

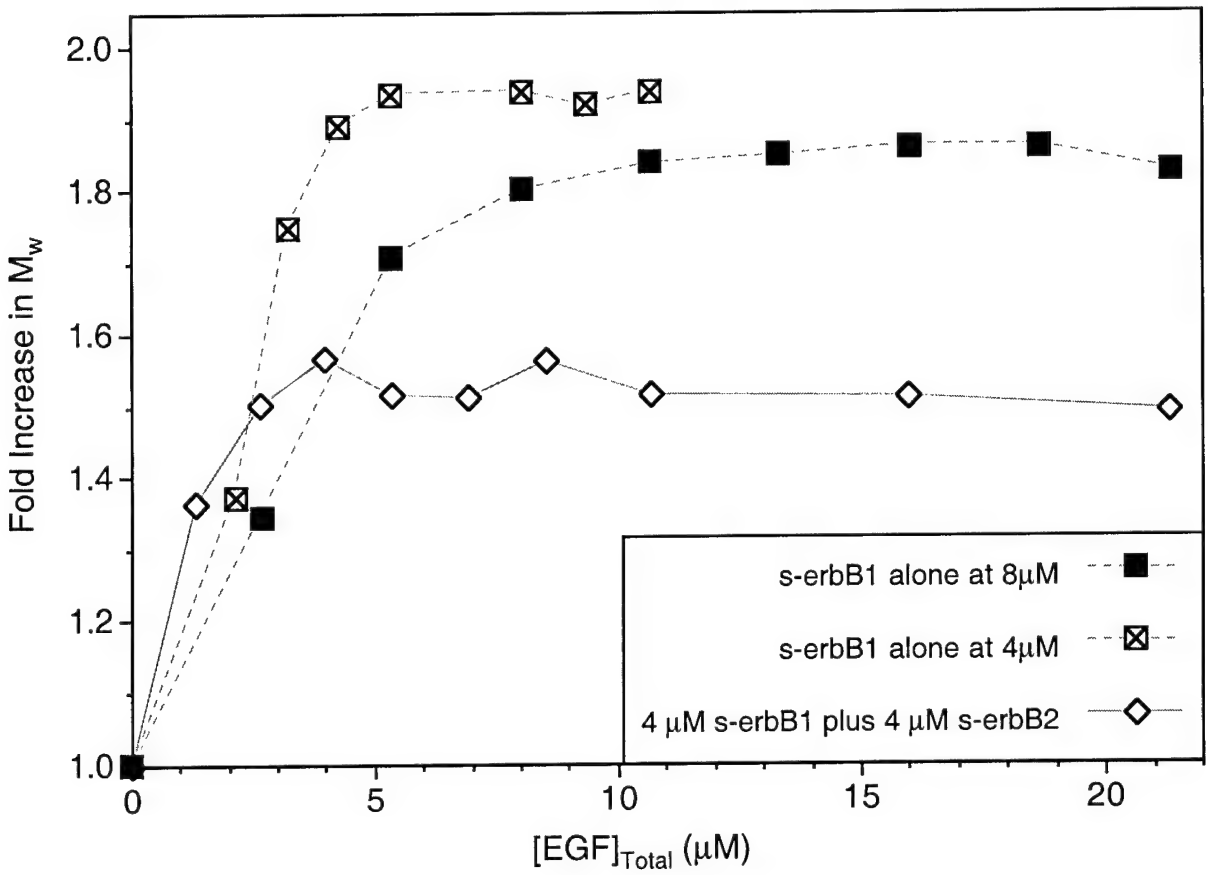


Figure 5
MALLS studies demonstrate that EGF induces complete homodimerization of s-erbB1, but does not induce formation of heterodimers between s-erbB2 and s-erbB1. The weight-averaged molecular mass (Mw) of the s-erbB1/s-erbB2 mixture increases such that after addition of one EGF molecule for each s-erbB1 molecule in the mixture, Mw reaches a maximum coincident with all s-erbB1 forming homodimers, and s-erbB2 remaining monomeric.

Figure 5 presents our MALLS studies, which show that EGF is not able to induce the expected extracellular domain heterodimerization. MALLS monitors the weight-averaged molecular mass ($M_{\overline{w}}$) of the particles in solution. For a sample containing only 4 μM s-erbB1, $M_{\overline{w}}$ is doubled upon addition of greater than 4 μM EGF (Figure 5 crossed squares). Similarly, for a sample containing 8 μM s-erbB1, $M_{\overline{w}}$ is almost doubled when more than 8 μM EGF is added (Figure 5 filled squares). By contrast, for a sample containing 4 μM s-erbB1 plus 4 μM s-erbB2, $M_{\overline{w}}$ reaches a maximum value when EGF is added to a final concentration of 4 μM (Figure 5, open diamonds). The fold-increase in $M_{\overline{w}}$ in this case is only 1.5. This is the expected result if EGF induces s-erbB1 homodimerization while s-erbB2 remains monomeric in the mixture. $M_{\overline{w}}$ is defined:

$$\overline{M}_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} \qquad \text{for } n \text{ moles of } i \text{ different species with molecular mass } M_i$$

In a sample containing 4 μM monomeric s-erbB2 (80 kDa) plus 2 μM s-erbB1 dimers (160 kDa), $M_{\overline{w}}$ would be estimated as 120 kDa, or a 1.5-fold increase over the monomeric $M_{\overline{w}}$ of 80 kDa. Thus, the

MALLS data provide no evidence for EGF-induced s-erbB1/s-erbB2 heterodimerization, and argue that s-erbB1 in the mixture homodimerizes with s-erbB2 as a monomeric "bystander".

These results suggest that the ligand-induced erbB1/erbB2 hetero-oligomers observed by several groups must form through a mechanism distinct from that used by erbB1 for homodimerization upon EGF binding. In the homomeric case, the isolated extracellular domain can recapitulate the interaction. In the heteromeric case it cannot. However, one caveat to this finding is that we have no independent validation of the functional quality of our s-erbB2 preparations. Since erbB2 has no known ligand, we cannot validate the protein by virtue of its ability to bind ligand, as was done for s-erbB1, s-erbB3, and s-erbB4 (Figure 2). However, we do not believe that the s-erbB2 is non-functional, since it is able to form NRG1-induced heterodimers with both s-erbB3 and s-erbB4 (see below). Furthermore, MALLS studies of potential heterodimers formed between s-erbB1 and s-erbB3 or s-erbB4 (with EGF or NRG1-β1) also gave clear negative results (not shown). Thus, in spite of its ability to form EGF-induced homodimers, s-erbB1 does not form heterodimers with other isolated erbB receptor extracellular domains.

- *confirm findings regarding ligand-induced s-erbB homo- and heterodimerization in vitro using analytical ultracentrifugation (month 1-18)*

Using sedimentation analytical ultracentrifugation and multi-angle laser-light scattering (MALLS), we have studied ligand-induced dimerization of each s-erbB protein. Figure 6 shows typical results from sedimentation equilibrium experiments (6,000 rpm) in which samples of each s-erbB protein were centrifuged both with and without the most relevant growth factor ligand. Inspection of the raw centrifugation data in Figure 6 shows that addition of a 2-fold molar excess of EGF to s-erbB1, or of a 2-fold excess of NRG1-β1 to s-erbB4 (filled symbols) results in a radial distribution indicative of a species larger than the s-erbB protein monomer. Since the s-erbB proteins have molecular masses of around 85 kDa, and the added ligands have molecular masses of only 6.3 kDa (EGF) and 8.3 kDa (NRG1-β1) , this can only be explained if the s-erbB protein is induced to oligomerize upon addition of the growth factor. Data fitting assuming a single ideal species for the ligand-free receptor gives molecular masses of 80 kDa and 97 kDa for s-erbB1 and s-erbB4 respectively. The best fit to the ligand/receptor mixture is obtained with two ideal species representing excess ligand and the s-erbB dimer. The residuals for these fits (experimental value minus fit value), plotted above the data in Figure 6, are both small and random, indicative of good fits to the data.

By contrast with the case for s-erbB1 and s-erbB4, addition of excess ligand to s-erbB2 or s-erbB3 results in a radial distribution consistent with a single species that is smaller than monomeric s-erbB protein. This is the expected result if the s-erbB protein does not bind ligand, or does not oligomerize upon ligand binding, since the distribution is now contributed to by free ligand that is 10-fold smaller (6 to 8 kDa) than the s-erbB protein (approx. 85 kDa). While best fits to a single ideal species gave molecular masses of 80.8 kDa and 84 kDa for s-erbB2 and s-erbB3 (without ligand) respectively, best fits to the ligand/receptor mixtures were obtained with two ideal species representing excess ligand and the s-erbB monomer (77.5 and 85 kDa for s-erbB2 and s-erbB3 respectively). Again, residuals for these fits are plotted above the data in Figure 3, and are both small and reasonably random, suggesting reasonable fits.

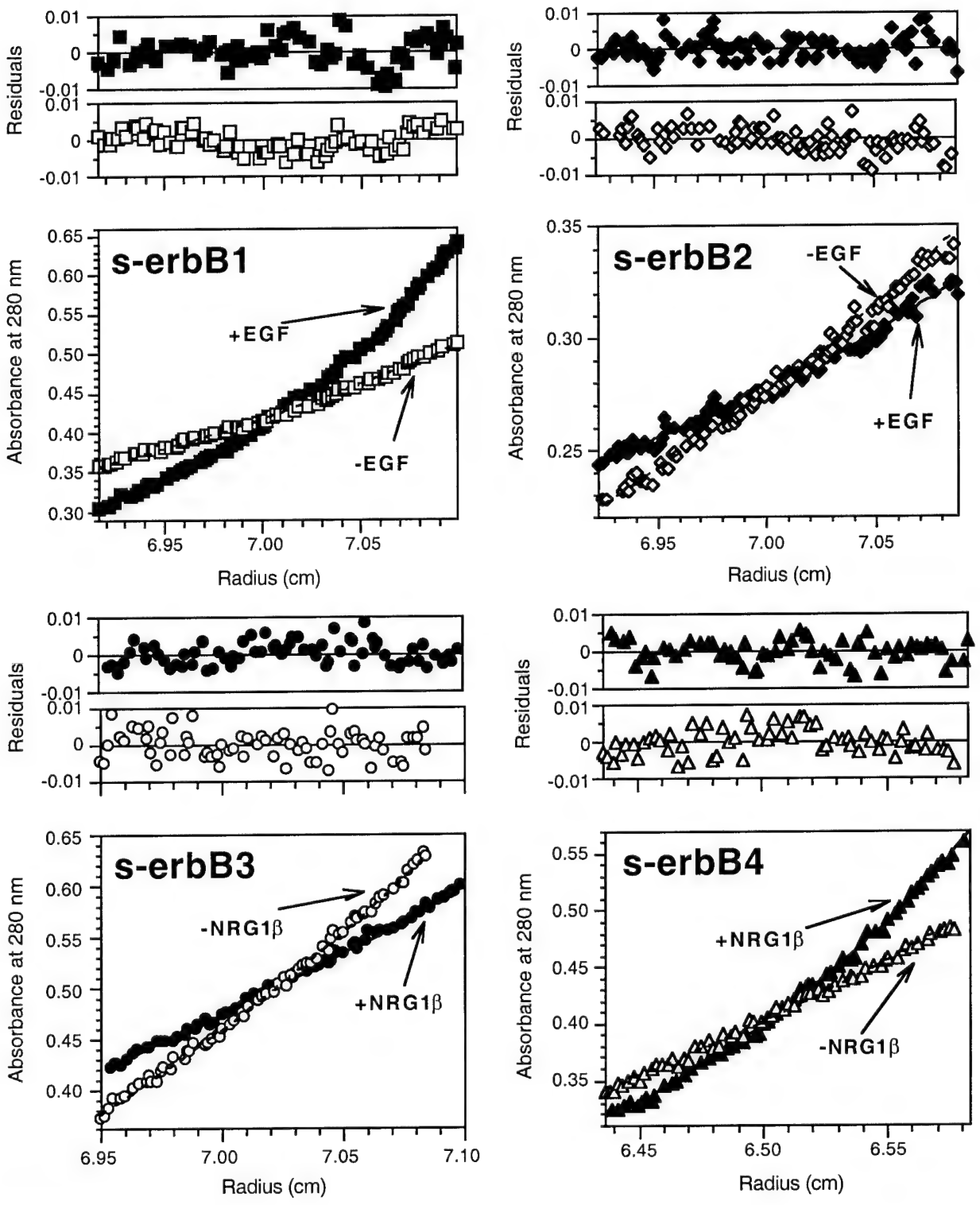


Figure 6
Representative sedimentation equilibrium analytical ultracentrifugation data for analysis of s-erbB homodimerization induced by EGF or NRG1-β1. In each case, open symbols represent the unliganded receptor, which is fit as an ideal single species (molecular mass range from 80 kDa for s-erbB1 to 97 kDa for s-erbB4). Filled symbols represent samples to which has been added a two-fold molar excess of the noted ligand. Fits to these data are with two ideal species - fixing the Mw of the ligand and floating the Mw of the complex. Fits return Mw of 198 kDa for s-erbB1 (dimer), 77.5 kDa for s-erb2 (monomer), 85 kDa for s-erbB3 (monomer), and 197 kDa for s-erbB4 (dimer). As described in the text, this result is clear from inspection of the

curves. All experiments shown were performed at 6,000 rpm (other speeds giving the same result). Residuals for the fits described are shown, and are both small and random, indicative of a good fit.

These data confirm the MALLS finding that, while s-erbB1 and s-erbB4 dimerize upon binding to EGF and NRG1-β1 respectively, s-erbB3 does not dimerize when it binds NRG1-β1. The inability of EGF to induce s-erbB2 dimerization is consistent with the lack of significant binding (Figure 2). NRG1-β1 also failed to bind s-erbB2 or to induce its dimerization (Figure 2 and 9). In other sedimentation equilibrium experiments (not shown) we found that s-erbB1 dimerization is also induced by TGF-α and HB-EGF (to which it binds), but not by NRG1-β1 (to which it does not bind). Dimerization of s-erbB4 was seen with NRG1-β1 (to which it binds), but not with HB-EGF, or EGF (to which it does not bind in our hands). Thus, all ligands tested that are capable of binding to s-erbB1 or s-erbB4 also induce their homodimerization. The failure of neuregulins to induce homodimerization of s-erbB3 represents the only example we have seen in which binding is observed in the absence of associated homodimerization. Previous studies with full-length NRG1-β1 (Horan *et al.*, 1995) also showed strong binding to s-erbB3 in the absence of induced dimerization.

Also using analytical ultracentrifugation, we have confirmed the inability of EGF to induce s-erbB1/s-erbB2 heterodimerization (Figure 7), and the failure of NRG1-β1 to induce s-erbB1/s-erbB4 heterodimerization (Figure 8). Figure 7 shows plots of the natural logarithm of absorbance against $(r^2 - r_o^2)/2$, where r is the radial position in the sample, and r_o the radial position of the meniscus for datasets collected at 6,000 rpm. For an ideal single species this plot is linear, with a gradient $(M\omega^2(1-V_2\rho)/RT)$ that is proportional to the molecular mass (M) of the ideal species. The lines obtained for s-erbB1 alone and s-erbB2 alone are approximately the same, and yield molecular masses of 80.5 and 78.9 kDa respectively. Addition of one equivalent of EGF to s-erbB1 results in near doubling of the gradient of this line, consistent with the ability of EGF to induce complete dimerization of s-erbB1. By contrast, addition of EGF to a 1:1 mixture of s-erbB1 and s-erbB2 increases the gradient of the line to only an intermediate extent (1.3-fold) and causes a greater deviation from linearity. This result is consistent with the interpretation of MALLS studies (Figure 5) that EGF induces homodimerization of s-erbB1 in the s-erbB1/s-erbB2 sample while the s-erbB2 remains monomeric.

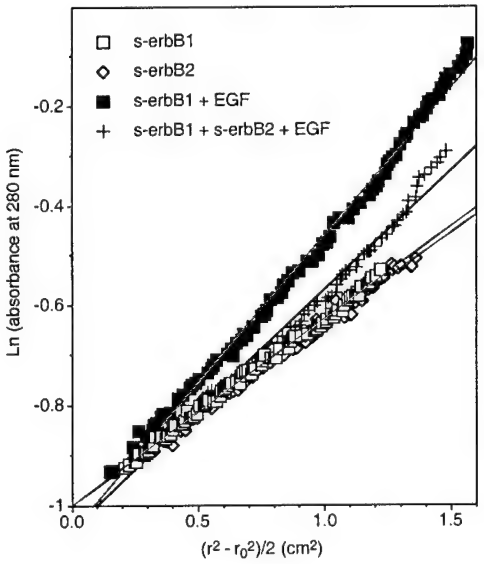


Figure 7
Plots of the natural logarithm of absorbance against the radius squared for analytical ultracentrifugation data. S-erbB1 and s-erbB2 alone yield straight lines in this plot, with gradients proportional to their molecular mass. Addition of EGF to s-erbB1 doubles the gradient showing that dimerization results. For an s-erbB1/s-erbB2 mixture, addition of a 2-fold excess of EGF yields an increase in the gradients of only about 1.3-fold. As in Fig 5, this is expected is the s-erbB1 homodimerizes while s-erbB2 remains monomeric.

These experiments, together with chemical crosslinking studies and co-immuno-precipitation studies (not shown), confirm the MALLS finding that EGF is not able to induce heterodimerization of the isolated extracellular domains of erbB1 and erbB2.

Heterodimerization of erbB1 and erbB4 upon treatment with EGF or NRG has been reported by several groups (*e.g.* Cohen *et al.*, 1996; Zhang *et al.*, 1996). Since we have been able to show that s-erbB1 and s-erbB4 both bind to their relevant ligands and homodimerize efficiently (Figures 3 and 6), we can be confident that both of these proteins are functionally active. We next used analytical ultracentrifugation to determine whether s-erbB1 and s-erbB4 can form heterodimers upon treatment with EGF or NRG1-β1

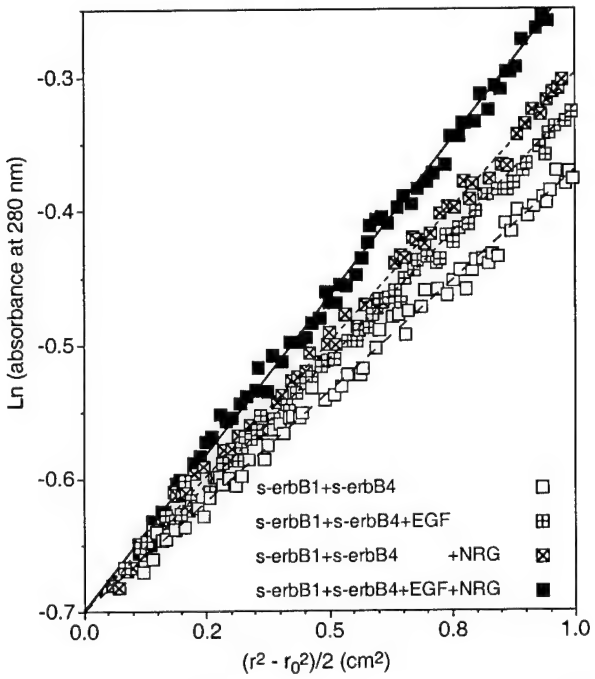


Figure 8
Analytical ultracentrifugation data, presented as ln(Abs) against r^2 plots, for s-erbB1/s-erbB4 heterodimerization. The s-erbB1/s-erbB4 mixture without ligand gives a straight line with gradient that yields monomer molecular mass. Addition of one molar equivalent (to total receptor) of EGF alone, or of NRG alone results in an increase in molecular mass consistent with homodimerization of one species only. Addition of both EGF and NRG at the same level results in a substantial increase in the gradient, indicating that both species are homodimerizing independently (see text for explanation).

Figure 8 presents ln(Abs) against $(r^2-r_o^2)/2$ plots for a series of 1:1 s-erbB1/s-erbB4 mixtures. The total receptor concentration is the same in each case. With no added ligand, the gradient of the straight line (proportional to molecular mass when an ideal single species is considered) gives an average monomeric molecular mass of approximately 80 kDa when divided by the appropriate constants. Addition of EGF to a concentration twice that of total receptor (*i.e.* 2 EGF molecules per s-erbB1 plus 2 EGF molecules per s-erbB4) increases the gradient of the straight line by a factor of approximately 1.3, suggesting that some dimerization is induced. Addition of NRG1-β1 to the same final concentration gives a similar result. Note that ligand is not limiting in either of these cases, suggesting that the limited increase in gradient results from homodimerization of only s-erbB1 when EGF is added, and only s-erbB4 when NRG1-β1 is added. If this is true, then an identical sample containing the same total ligand concentration, but as a mixture of EGF and NRG1-β1 should show substantially more dimerization, as both s-erbB1 and s-erbB4 will be capable of homodimerizing independently. The steepest line in Figure 8 shows this to be the case, providing evidence that, as with s-erbB1 and s-erbB2, hetero-dimerization of s-erbB1 and s-erbB4 does not occur under these conditions - with either EGF or NRG1-β1. In similar experiments we have shown that s-erbB1 also fails to form heterodimers with s-erbB3, regardless of whether EGF or NRG1-β1 is added. Thus, we have failed to detect formation of any heterodimer that includes s-erbB1.

Hetero-oligomerization between NRG-binding receptors and s-erbB2

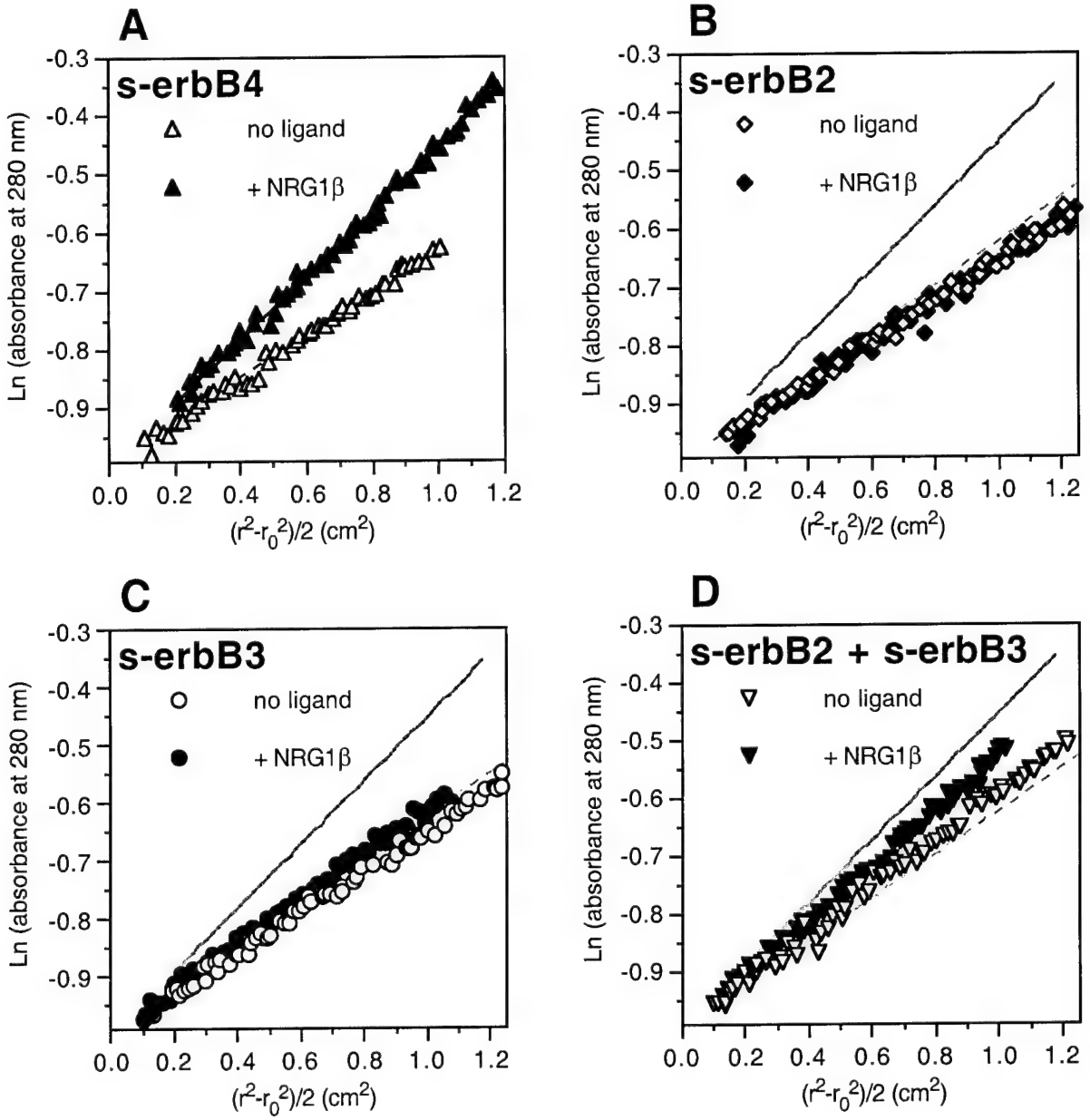
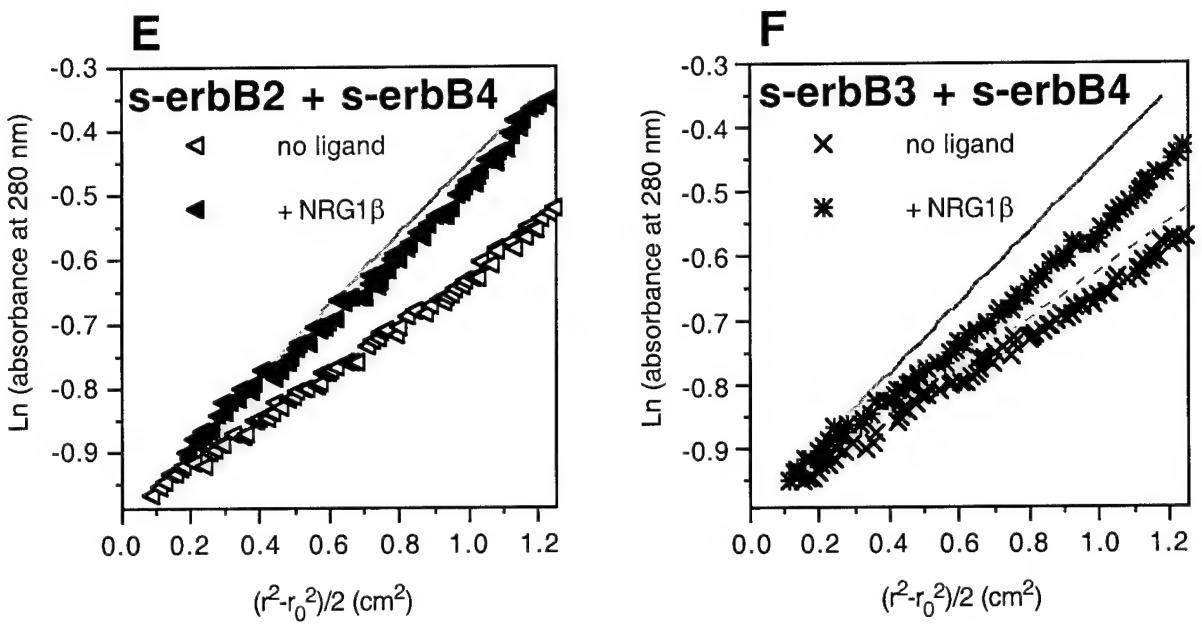


Figure 9
Analytical ultracentrifugation evidence for formation of s-erbB2/s-erbB3 and s-erbB2/s-erbB4 heterodimers. Panel A shows the increase in gradient of the ln(Abs) against r^2 plot that results from NRG1-β1-induced s-erbB4 homodimerization. These lines are superimposed in grey on all other panels. Panels B and C show that NRG1-β1 fails to induce homodimerization of s-erbB2 or s-erbB3, while panels D and E demonstrate that NRG1-β1 can induce formation of s-erbB2/s-erbB3 and s-erbB2/s-erbB4 heterodimers. Panel F indicates that s-erbB3 and s-erbB4 do not heterodimerize efficiently upon NRG1-β1 binding.



Using a similar approach to that used for investigating s-erbB1/s-erbB2 and s-erbB1/s-erbB4 heterodimerization, we next tested the ability of s-erbB3 and s-erbB4 to heterodimerize with one another and with s-erbB2. Recent studies by Sliwkowski's laboratory (Fitzpatrick *et al.*, 1998; Jones *et al.*, 1999) have shown that dimeric IgG fusion proteins containing s-erbB2 together with either s-erbB3 or s-erbB4 have an enhanced affinity for NRG compared with those containing just s-erbB3 or just s-erbB4. This finding has been interpreted to suggest that s-erbB2/s-erbB3 and s-erbB2/s-erbB4 heterodimers represent high-affinity NRG receptors. In Figure 9, ln(Abs) versus radius^2 plots are shown for each pairwise combination of s-erbB2, s-erbB3, and s-erbB4 with and without NRG1-β1. In panel A, NRG-induced homodimerization of s-erbB4 is clearly seen by the approximately 1.6-fold increase in gradient upon addition of a 2-fold excess of NRG1-β1. No such increase is seen upon addition of NRG1-β1 to s-erbB2 (panel B) or s-erbB3 (panel C). In panel D it can be seen that NRG1-β1 induces a small increase in the gradient of the ln(Abs) against r^2 line for an s-erbB2/s-erbB3 mixture. The increase is less dramatic than that seen for s-erbB4, and there may be some tendency for these two s-erbB proteins to interact with one another in the absence of ligand (the gradient is slightly greater than that for the ligand-free mixture than for any unliganded s-erbB protein). However, the NRG-induced shift in gradient shown here is reproducible, and suggests some weak ligand-induced heterodimerization of s-erbB2 and s-erbB3. Comparing panel F with Figures 7 and 8, it can be seen that the s-erbB3/s-erbB4 mixture behaves, when NRG1-β1 is added, in the same way as an s-erbB1/s-erbB4 mixture with NRG1-β1 or either a s-erbB1/s-erbB2 or s-erbB1/s-erbB4 mixture when EGF is added. In other words, addition of NRG1-β1 appears to induces s-erbB4 homodimerization while s-erbB3 remains monomeric. There is therefore no evidence for s-erbB3/s-erb4 heterodimerization. However, panel E of Figure 9 appears almost identical to panel A, despite the fact that the mixture contains s-erbB2 that neither binds NRG1-β1 nor is induced to dimerize by this ligand. The only explanation for this result is that NRG1-β1 induces efficient heterodimerization of s-erbB2 and s-erbB4. This finding allays our fears that negative results with s-erbB1/s-erbB2 heterodimerization simply reflect a non-functional s-erbB2 preparation.

Thus, all of the subtasks of Task 1 have been completed, and their findings (outlined above) were published in the *EMBO Journal* in September 2000 in a manuscript entitled "Extracellular Domains Drive Homo- but not Hetero-Dimerization of ErbB Receptors." By Kathryn M. Ferguson, Paul J. Darling,

Timothy L. Macatee, Mohita M. Mohan, and Mark A. Lemmon (*EMBO J.* **19,** 4632-4643). A reprint of this paper is attached in the Appendix.

Task 2.
To test the prediction of studies using s-erbB proteins in a cellular context.

Our original precise approach to Task 2 was described as follows in the application for this grant:

- from the studies of s-erbB heterodimerization, we will predict which combinations of erbB receptors will create high-affinity binding sites for which ligands. These predictions will be tested by studying ligand binding to breast cancer cell-lines that express known combinations of erbB receptors (months 18-24).

Since the findings from Task 1 argued against the simple heterodimerization model that prevails in the literature, this question becomes irrelevant in the context proposed. Since no heterodimers were detected between erbB receptor ectodomains with distinct specificities, predicting which combinations of erbB receptors create high-affinity binding sites for which ligands becomes trivial, and testing these predictions can be achieved by inspection of the literature. Therefore, for precisely the same task, but with different focus, it becomes important to determine which heteromeric complexes of full-length erbB receptors are induced to form by which erbB ligand. We have focused on this question.

Thus, instead of testing in living cells which combinations of erbB receptors create high-affinity binding sites for which ligands, we have simply inverted the question to ask which combinations of erbB receptors are induced to self-associate by which ligands. We initiated development of several approaches to address this question, several of which were unsuccessful.

One approach, however, has allowed us to begin to ask which erbB receptor heteromers form in living cells, and our preliminary data suggest that it has great promise. The approach takes advantage of the properties of the interleukin-2 (IL2) receptor to report on erbB receptor homo- and hetero-oligomerization in different cell-lines. The IL2 receptor (IL2-R) has three components: an α-chain, a β-chain, and a γ_c chain (Nelson and Willerford, 1998). Depending on the cell-type, an IL2 response (at least a proliferative response) can be reconstituted simply by inducing homodimerization of the IL2-R β-chain (in BA/F3 cells), or heterodimerization of the IL2-R β and γ_c chains (required in 32D and CTLL2 cells) (Nakamura et al., 1994; Nelson et al., 1994). The α-chain is not required for signaling. Nelson *et al.* (Nelson et al., 1994) were able to generate functional chimeric proteins in which the extracellular domains (ECD's) of the IL2-R β- and γ_c- chains were replaced with the ECD from Kit, a tyrosine kinase receptor for stem cell factor (SCF). SCF is well known to induce homodimerization (and no larger oligomers) of the Kit ECD (Lemmon et al., 1997; Philo et al., 1996). In BA/F3 cells (which do not normally respond to SCF), expression of a chimera with the Kit ECD and IL2-R β intracellular domain (Kit/β) is sufficient to allow SCF-induced cell proliferation. In 32D myeloid progenitor cells or CTLL2 cells (an IL2-dependent T-cell line), homodimerization of the Kit/β chimera is not sufficient for a proliferative response. Rather, in these cells, Kit-induced heterodimerization of a Kit/β and a Kit/γ_c chimera was necessary for a proliferative response. Thus, this system provides a convenient potential means for analyzing homodimerization (using β-chain chimerae in BA/F3 cells) and heterodimerization (using β- and γ_c-chain chimerae in 32D or CTLL-2 cells) using proliferation assays.

In addition to their utility as reporters for IL2-R signaling, BA/F3, 32D, and CTLL-2 cells provide an excellent experimental system for analyzing erbB receptor interactions, since these cells do not ordinarily express members of this receptor family (except for very low erbB3 levels detected by

Northern blotting in BA/F3 cells (Riese et al., 1995)). We therefore chose to adapt the approach of Nelson *et al.* (Nelson et al., 1994) for the analysis of *in vivo* erbB receptor interactions. We therefore generated chimerae in which the ECD is from erbB1 or erbB2, while the intracellular domain comes from the IL2-R β- or γ_c-chain. Depending on the construct, the transmembrane domain (TMD) is derived from the erbB receptor or the IL2-R chain in the chimera. The chimerae are named B1/B1/β, B1/β/β, B2/γ/γ, etc., where B1/B1/β, for example, corresponds to a chimera in which the ECD and TMD are from erbB1, while the intracellular domain comes from the IL2-R β-chain.

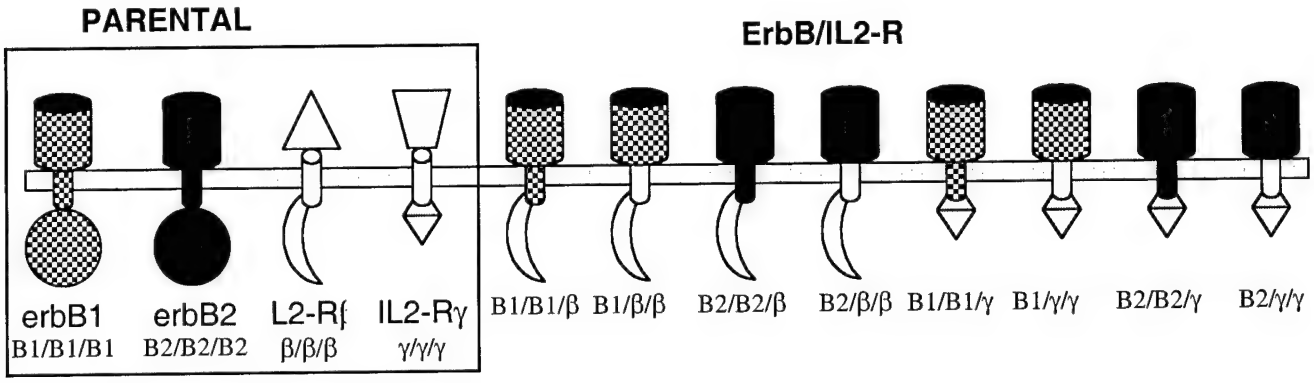


Figure 10
Chimerae containing erbB1 or erbB2 EC or TM domains, plus IL2-R β- or γ-chain intracellular domains. The parental receptor chains are shown in the box at left of the figure, and are shaded (erbB1 is checkered, erbB2 is black, IL2-Rβ is white, and IL2-R γ is gray). Each portion of the chimerae is shaded according to its origin.

As a first step towards establishing this system for analysis of erbB receptor interactions, we analyzed the ability of BA/F3 cells expressing B1/B1/β or B1/β/β to proliferate without IL3 (on which they are normally dependent for growth), but with EGF. The chimera (in pcDNA3.1neo) was electroporated into BA/F3 cells, and transfected cells were selected in medium containing 0.5 mg/ml G418. Fluorescence-activated cell sorting (FACS) was then used to select cells with the erbB1 ECD at their cell-surface (parental BA/F3 cells have no erbB1). FACS analysis of the resulting pool is shown in Fig. 11. The majority of cells in the pool express the chimera (in this case B1/B1/β), although expression levels are relatively low (fewer than 10,000 molecules by comparison with other erbB1-expressing cells under the same conditions).

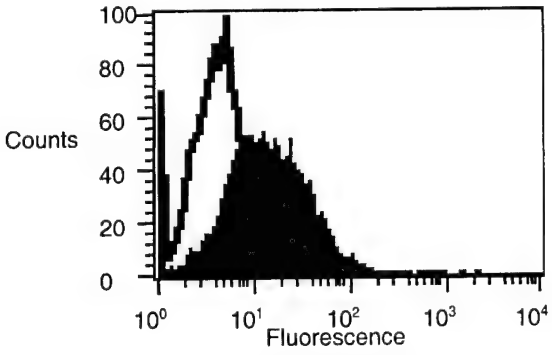


Figure 11
FACS (BD FACStar)analysis of BA/F3 cells expressing B1/B1/β (filled trace) and parental BA/F3 cells (open trace), detected using phycoerythrin-labeled anti-erbB1 antibody EGFR.1 (BD Pharmingen).

The normal IL3-containing medium of the cells was replaced for 24 h with test medium, and cell proliferation was assessed for the last 4 h of this incubation using a ^{3}H-thymidine incorporation assay.

As shown in Fig. 12, EGF induces proliferation of cells that express the B1/B1/β or B1/β/β chimerae, but not of parental BA/F3 cells. The response of the B1/β-expressing cells to saturating

EGF was approximately equal to that elicited by 1pg/ml of IL3 in parental BA/F3 cells. This level of response provides a robust signal in our assay. Possible reasons for the difference between maximal EGF-induced proliferation in this system and maximal IL3-induced proliferation include the relatively low level of B1/β chimera expression (Fig. 11), and the possibility that EGF-induced homodimerization of the IL2-R β-chain in the B1/β chimera does not have optimal geometry.

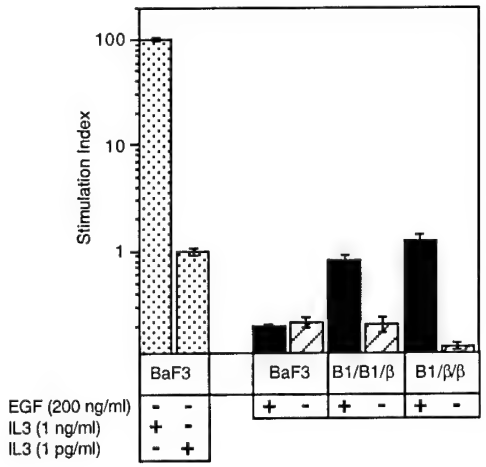


Figure 12
BA/F3 cells expressing the B1/B1/β or B1/β/β chimerae, but not parental BA/F3 cells proliferate in response to EGF. In a 96-well plate, 50,000 cells (per well) were treated for 24 h with 200 ng/ml EGF or 1 ng/ml IL3. For the final 4 h of incubation at 37°C, cells were incubated with 1 μCi of ^{3}H-thymidine. Cells were then harvested onto a glass fiber filter , washed to remove unincorporated ^{3}H-thymidine, and ^{3}H-incorporation was measured by liquid scintillation counting of cells harvested from the plate using a Wallac 1450 Microbeta counter. Each result represents the mean (± standard deviation) of at least 4 repeats. The 'stimulation index' (plotted on a log-scale) represents the percentage of counts measured (saturating IL3 gives 100%).

Fig. 12 shows that the B1/B1/β and B1/β/β are approximately equivalent in their ability to promote EGF-dependent BA/F3 cell proliferation. FACS analysis indicated that the level of expression (and homogeneity of expression levels) in the two transfectant pools were also very similar. Thus, the TMD's of erbB1 and IL2-R β are equivalently able to support oligomerization of the B1/β chimerae.

We have also generated erbB2/IL2-R β-chain chimerae, as listed in Fig 10. These chimera appear to be expressed at higher levels in BA/F3 cells than the B1/β chimerae (after normalizing for antibody differences). Consistent with our expectations, neither B2/B2/β nor B2/β/β supports a proliferative response to EGF or NRG's in transfected BA/F3 cells (not shown). However, when B2/β/β is expressed alongside B1/γ/γ in BA/F3 cells, EGF can induce proliferation (Fig. 13), although B1/γ/γ alone is not sufficient for this response.

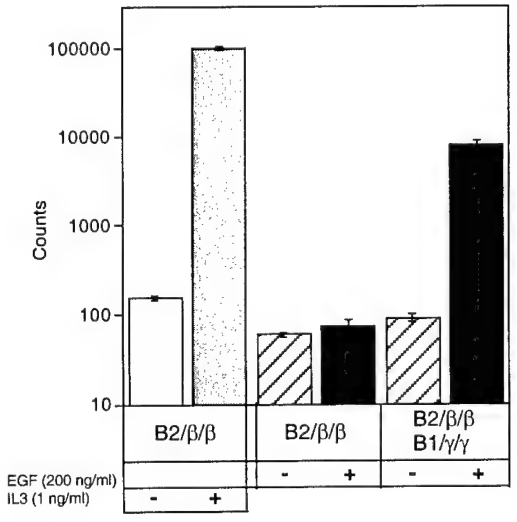


Figure 13
BA/F3 cells expressing both B2/β/β and B1/γ/γ, but not B2/β/β alone (or B1/γ/γ alone) proliferate in response to EGF. Details are as for Figure 12, but data are plotted as ^{3}H 'counts' rather than as 'stimulation index'.

Thus, this approach has provided clear evidence in vivo that erbB1 and erbB2 do interact with one another to drive association of the β-chain and γ-chain chimerae. Work is now in progress to investigate all other pairwise interactions between IL2-R chimerae, induced by each of the different erbB ligands. This work will be continued following the end of the funding period for this grant to address the critical question posed in Task 2.

It is important to note that the 'readout' used in these studies is quite different from that employed for full-length erbB receptors in the same cell-lines by Stern and colleagues (Riese et al., 1996) or Yarden and colleagues (Pinkas-Kramarski et al., 1996). In those studies, the ability of the full-length erbB receptor tyrosine kinase to stimulate cell growth was being studied, yielding no distinction between homo- and hetero-oligomer formation. By contrast, we are using homo- or hetero-dimerization of the β- and γ-chains of the IL2 receptor as a direct 'read-out' for interactions between erbB receptors. The fact that we are using the same cell-lines as used for previous studies of full-length receptors is coincidental.

As a reporter for erbB receptor homodimerization in BA/F3 cells, our experience with the B1/β chimerae suggests that this approach will be very robust. If this system works for heterodimerization of B1/β and B1/γ chimerae as well as was reported by Nelson *et al.* (Nelson et al., 1994) for Kit chimera, this approach promises to be extremely powerful in a systematic analysis of *in vivo* ligand-induced erbB receptor interactions.

Thus, the question asked in Task 2 of the original Statement of Work was re-cast, having as its focus the determination of which erbB receptor hetero-oligomers each erbB ligand could induce *in vivo*. To address this question, new methodology was required, which we have succeeded in developing utilizing chimerae made with IL2 receptors. This approach appears to be successful in our experiments to date, and has allowed us to demonstrate that a construct containing just the extracellular domain of erbB1 can homodimerize in vivo, as we have previously shown *in vitro* (see above). Most interestingly, whereas isolated extracellular domains of erbB1 and erbB2 cannot form heterodimers *in vitro* when exposed to EGF, our studies with IL2-R chimerae demonstrated that constructs containing only the extracellular domains of erbB1 and erbB2 can form an EGF-dependent heteromeric complex *in vivo*. We hypothesize that this complex is larger than a dimer, and requires cooperation of multiple weak interactions that can only be achieved when the receptors are effectively concentrated by restriction to two dimensions. Further investigation of this is ongoing.

To summarize with our new approach, Task 2 is only partly completed, although we feel that substantial progress has been made. Future experiments promise to determine which oligomers will form, what the size of the oligomers are, etc. These experiments will provide further insight into mechanistic aspects of erbB receptor heteromerization.

Task 3.

To determine which erbB receptor hetero- or homodimers are responsible for the antiproliferative effects seen for certain erbB ligands on breast cancer cell-lines.

The subtasks defined in the original grant proposal were as follows:

- study the proliferative and antiproliferative effects of different erbB ligands on selected breast cancer cell-lines, starting with the effect of NRG's on SKBR3 and MDA-MB-453 cells. Analyze effects on proliferation and differentiation at different dose levels, and attempt to correlate with the results obtained in Task 1 to determine which hetero or homo dimers are responsible for anti-proliferative effects, and which are responsible for proliferative effects.

(months 24-36)

- attempt to generate mutated ligands that exclusively induce formation of the anti-proliferative hetero- or homodimer, and ligands that selectively inhibit formation of the proliferative hetero- or homodimer (months 30-36 and beyond)
- test the designed ligands on a panel of breast cancer cell-lines (months 30-36 and beyond)

Our findings in attacking Tasks 1 and 2 argue strongly against the simple idea that different erbB ligands elicit different cellular responses because they are bivalent and have different pairwise combinations of receptor binding sites that can drive formation of different (ligand-specific) erbB receptor heterodimers. The fact that our *in vitro* studies with extracellular domains fail to detect most of the heterooligomers that can be seen *in vivo* with full-length receptors, yet recapitulated erbB1 and erbB4 homodimerization, argues that there is a mechanistic difference between ligand-induced homo- and hetero-mer formation. With our initial simple mechanistic hypothesis invalidated, the 2nd and 3rd subtasks defined above, with which we would have been occupied during the final 6 months of the grant period, are not accessible (or worthwhile) for study. Rather, our findings beg the question as to what precisely does drive erbB receptor hetero-oligomerization. Since hetero-oligomerization is the only mechanism available for erbB2 activation (since it has no ligand), understanding this mechanism is of primary importance in defining its role in breast cancer. We have therefore adapted the first subtask of Task 3 to address this question.

Our studies in Task 3 have analyzed the effects of different erbB ligands on selected breast cancer cell-lines as proposed in the original statement of work, starting with the effect of EGF and NRG's on SKBR3 and MDA-MB-453 cells. However, instead of analyzing effects on proliferation and differentiation at this stage, we have focused instead on more receptor-proximal events in order to determine which receptors are activated by each ligand, and thus which immediate downstream events are elicited. The primary aim of the approach is to determine what characteristics of a given activated receptor are required for it to be able to form hetero-oligomers with another receptor not activated by the same ligand. While there is substantial overlap in most of the downstream signaling pathways activated by the four erbB receptors, PI 3-kinase activation stands out as a response that is associated primarily (if not exclusively) with erbB3 (Fedi *et al.*, 1994; Kim *et al.*, 1994; Soltoff *et al.*, 1994), and that therefore provides a good reporter for erbB3 trans-activation through hetero-oligomer formation. PI 3-kinase activation requires recruitment of a p85/p110 PI 3-kinase heterodimer via SH2 domains in the p85 subunit. ErbB3 is the only erbB receptor that contains tyrosine phosphorylation sites in the sequence context recognized by the p85 SH2 domains (pYMxM). Several studies indicate that erbB ligands can only activate PI 3-kinase through erbB3 (Fedi *et al.*, 1994; Kim *et al.*, 1994; Soltoff *et al.*, 1994). Since erbB3 does not possess tyrosine kinase activity (Guy *et al.*, 1994; Kraus *et al.*, 1989; Sierke *et al.*, 1997), it cannot be activated directly by ligand-induced homodimerization, but rather can only be activated *in trans* through ligand-induced erbB receptor hetero-oligomerization. Thus, analysis of PI 3-kinase stimulation by erbB ligands provides a unique opportunity to study erbB receptor hetero-oligomerization events *in vivo* - monitoring downstream events.

To monitor PI 3-kinase activation *in vivo*, we take advantage of the properties of the pleckstrin homology (PH) domain from Grp-1 (general receptor of 3-phosphoinositides 1) (Klarlund *et al.*, 1997). This PH domain binds with very high affinity (K_d = 27 nM) and specificity to the major product of agonist-stimulated PI 3-kinase; phosphatidylinositol(3,4,5)trisphosphate (PtdIns(3,4,5)P_3) (Kavran *et al.*, 1998), and we recently determined its X-ray crystal structure (in complex with the PtdIns(3,4,5)P_3 headgroup) (Ferguson *et al.*, 2000). In serum-starved cells, a green fluorescent protein (GFP) fusion of

the Grp-1 PH domain (Grp1-PH) is seen by fluorescence microscopy to be diffusely localized in the cytoplasm and nucleus. However, when most breast cancer cell-lines are treated with EGF or NRG, the GFP/Grp1-PH fusion translocates rapidly to the plasma membrane. Many laboratories are now using this property of GFP fusion proteins with Grp1-PH and related PH domains to monitor PI 3-kinase activation in living cells (Gray *et al.*, 1999; Kavran *et al.*, 1998; Varnai *et al.*, 1999; Venkateswarlu *et al.*, 1998; Watton and Downward, 1999). Using this approach, we recently analyzed EGF- and NRG-induced translocation of GFP/Grp1-PH in a panel of breast tumor and medulloblastoma cell lines with characteristically different erbB receptor expression profiles. ErbB receptor expression profiles were obtained from literature reports (Beerli *et al.*, 1995; Beerli and Hynes, 1996; Daly *et al.*, 1997; Lewis *et al.*, 1993; Lewis *et al.*, 1996; Weiss *et al.*, 1997), and we have confirmed them by Western blotting and/or FACS analysis (not shown). They are summarized in the upper part of Fig. 14.

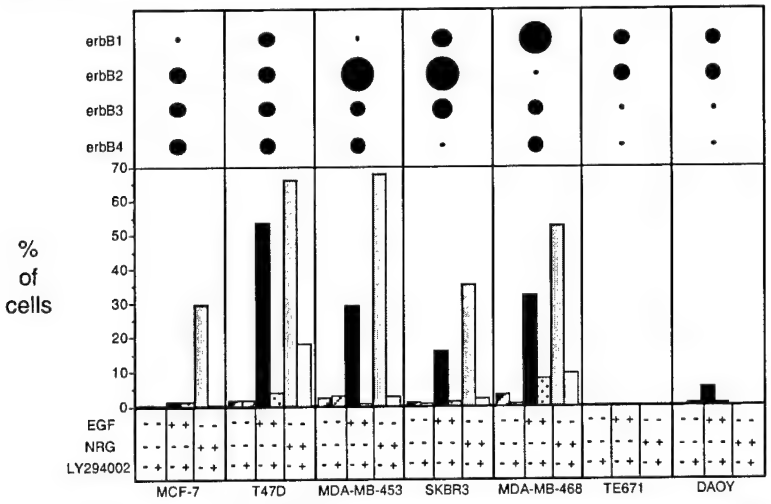


Figure 13
Analysis of PI 3-kinase activation upon treatment of breast cancer and medulloblastoma cells with EGF and NRG, reported as a percentage of (over 200 examined) cells in which a GFP/Grp1-PH fusion protein becomes translocated to the plasma membrane. Inhibition by the PI 3-kinase inhibitor LY294002 is also shown. In the top part of the figure, the black spots represent expression levels of the different erbB receptors. For example, MCF7 cells express moderate levels of erbB2, 3, and 4, but do not express erbB1.

In this assay, cells are transiently transfected with GFP/Grp1-PH fusion (in pEGFP-C1), and are then starved of serum overnight. Live cells are examined at 37°C by fluorescence microcopy in a MatTek glass-bottom dish on a heated microscope stage. EGF or NRG (or no ligand) is then added to 100 ng/ml, either alone or with the PI 3-kinase inhibitor LY294002, and the percentage of cells in which the GFP/Grp1-PH fusion has undergone translocation to the plasma membrane is counted. Translocation is maximal at about 5 minutes following stimulation. This procedure is repeated for several dishes, so that between 200 and 400 cells are examined. As shown in Fig. 14, treatment of T47D, SKBR3, and MDA-MB-468 breast cancer cells with either EGF or NRG results in a robust PI 3-kinase response. This is expected, since these cell-lines all express significant amounts of erbB1 and erbB3. By contrast, in MCF-7 cells, which lack erbB1, NRG but not EGF activates PI 3-kinase. Surprisingly, another cell-line reported not to express erbB1 (MDA-MB-453) did show a PI 3-kinase response to EGF. We initially considered that this might reflect the ability of erbB2/erbB3 heteromers to acting as 'surrogate' receptors for EGF, as suggested by several groups (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). However, FACS analysis of MDA-MB-453 cells indicated that they do express erbB1 at detectable (albeit low) levels. Since MCF-7 cells express both erbB2 and erbB3, yet do not respond to EGF, we consider that the difference between the EGF responsiveness of these cell-lines is most likely explained by differences in erbB1 levels (none in MCF-7 cells, low but detectable levels in 453 cells). The results in Fig 14 for the medulloblastoma cell-lines, TE671 and DAOY, show that erbB1 alone is not sufficient for EGF to activate PI 3-kinase, supporting the idea that erbB3 *trans*modulation by erbB1 is required to activate this pathway. We are currently investigating the ability of erbB3 expression in TE671 cells to reconstitute EGF-induce PI 3-kinase signaling.

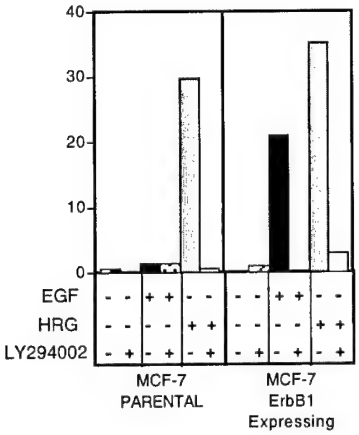


FIGURE 14
Translocation of a GFP/Grp1-PH fusion protein in response to EGF and NRG treatment, for both parental MCF-7 cells (left) and a stable MCF-7 cell line that expresses wild-type human erbB1. The percentage of cells in which a GFP/Grp1-PH fusion was translocated to the plasma membrane upon EGF- or NRG-treatment was analyzed as described for Fig 8. ErbB1 overexpression reconstitutes a PI 3-kinase response to EGF, allowing studies (discussed in Aim 3a) of the erbB1 requirements for erbB3 *trans*modulation.

We have recently generated stable cell-lines from MCF-7 cells that do express erbB1, and have confirmed expression by FACS and Western blotting. As shown in Fig. 15, MCF-7 cells that express erbB1 display a robust PI 3-kinase response to EGF. This response results from *trans*modulation of erbB3 by erbB1 that we have introduced into MCF-7 cells, providing us with a unique opportunity to define which of characteristics of erbB1 are required for its ability to *trans*-activate erbB3. We have subsequently shown, most interestingly, that a construct containing only the extracellular plus transmembrane domains of erbB1 is sufficient to confer upon MCF7 cells the ability to have PI 3-kinase activated by EGF. The following table summarizes our PI3-kinase activation data for MCF7 derivatives:

	EGF	TGFα	HB-EGF	BTC	ARG
Parental MCF7 cells	-	-	+	+	-
MCF7 + erbB1 (full-length)	+	+	+	+	+
MCF7 + erbB1 (ECD plus TM)	+	+	+	+	-

Table 2: Summary of PI 3-kinase activation studies by different erbB ligands in MCF 7 cells expressing no erbB1, full-length erbB1, or a truncation mutant of erbB1 that contains only the extracellular and transmembrane domains. Significant (+) or no significant activation (-) activation of PI 3-kinase was determined using the Grp1-PH translocation assay outlined in the text.

According to these results, HB-EGF and BTC are able to activate PI 3-kinase without involvement of the EGF receptor (erbB1), is agreement with previous reports that they are 'bispecific' ligands that can also bind to and activate erbB4. In these cases, erbB3 is likely activated via erbB4. However, both EGF and TGFα require the presence of erbB1 for their ability to activate PI 3-kinase in MCF7 cells. A simple suggestion would be that they induce heterodimerization of erbB1 and erbB3, leading to erbB3 activation (by transphosphorylation by erbB1). However, since deletion of the erbB1 intracellular domain does not affect the ability of erbB1 to mediate EGF and TGFα induction of PI3-kinase activation, this is not likely to be correct. Our *in vitro* studies showed that EGF and TGFα induce dimerization of the erbB1 extracellular domain. Therefore, it is almost certain that they will induce dimerization of the truncated erbB1 mutant expressed here. In some way, despite lacking intracellular domains, this dimer can trans-activate erbB3. A possible model for this, discussed by Schlessinger in a recent review (Schlessinger, 2000), is depicted in Figure 15. We are currently investigating erbB2 activation in the same cell-lines, to determine whether this mechanism might operate for that transmodulation event also.

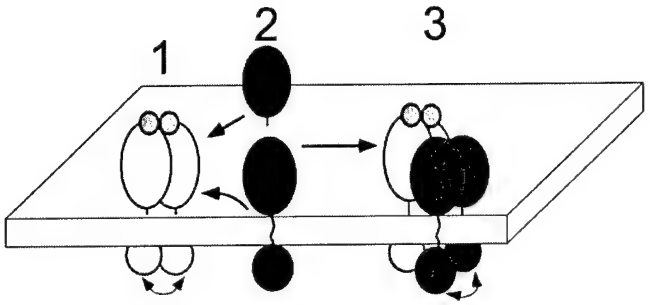


Figure 15
Model for transmodulation of erbB receptor family members by 'homodimer-nucleated heterotetramerization'. An EGF-induced activated erbB1 homodimer (**1**), which may or may not include extracellular domains, recruits two erbB3 (or other erbB) protomers (step **2**), which then activate one another by trans-phosphorylation within a hetero-tetramer (step **3**). Taken from Schlessinger (2000).

These findings are consistent with those of others, who have found that several aspects of erbB1 signaling are in fact independent of the kinase activity (Deb *et al.*, 2001; Hung *et al.*, 1998; Wright *et al.*, 1995), and support the idea that the relevant oligomer for erbB receptor transmodulation might be a tetramer or higher order oligomer rather than heterodimer. We are proceeding to test this hypothesis using a variety of approaches including those described here. In addition, these studies suggest that different ligands have different pathways to PI3 –kinase. The fact that amphiregulin requires erbB1 to activate PI 3-kinase in MCF7 cells, but unlike EGF requires the full-length receptor is consistent with recent work from Johnson's lab (Wong *et al.*, 1999), and is a target of our future studies.

EXPERIMENTAL PROCEDURES

Generation of s-erbB Constructs

A fragment of human erbB1 cDNA directing expression of residues 1-642 (1-618 of the mature sequence) followed by a hexahistidine tag and stop codon was subcloned into pFastBac1 (Life Technologies Inc). The 1955-base pair fragment was generated by PCR, introducing a unique *Bgl II* site immediately before the initiation codon and a unique *Xba I* site that follows the introduced stop codon. The 1955-base pair *Bgl II/Xba I* digested PCR product was ligated into *Bam HI/Xba I* digested pFastBac I. To minimize the risk of PCR artifacts, a 1260-base pair *Eco RI/Apa I* fragment of this PCR-derived clone was swapped for the equivalent region from the original erbB1 cDNA.

A fragment of human erbB2 cDNA directing expression of residues 1-647 (1-628 of the mature sequence) was generated similarly. In this case a unique *Xba I* site was introduced before the initiation codon, and a unique *Hind III* site was introduced to follow the histidine tag and stop codon. The 1980-base pair *Xba I/Hind III* digested PCR product was ligated into *Xba I/Hind III*-digested pFastBac I. An 1880-base pair internal fragment of this PCR product, extending from an *Nco I* site at the initiation codon to a unique *Sph I* site, was then swapped for the equivalent fragment from the original erbB2 cDNA.

Fragments encoding human erbB3 residues 1-639 (1-620 of the mature protein) and human erbB4 residues 1-649 (1-624 of the mature protein), with a unique *Bam HI* site at one end and *Xba I* site at the other, were generated by PCR, and ligated into *Bam HI/Xba I* digested pFastBac I. The sequence of all PCR-derived fragments and their cloning boundaries were confirmed by standard manual or automated dideoxynucleotide sequencing methods.

Protein Production

Typically 5 - 10 liters of Sf9 cells were grown as a suspension culture in Sf900-II medium (Gibco/BRL) using a number of 1 liter spinner flasks. Each 1 liter flask contained less than 500 ml of medium to ensure adequate aeration. When a cell density of 2.5×10^6 cells/ml (viability >98%) was

reached, freshly amplified high-titer virus stock was added to a multiplicity of infection (MOI) of approximately 5. Cultures were incubated for a further 96 hours. Clarified conditioned medium was then diafiltered against 3.5 volumes of 25 mM Tris-HCl, 150 mM NaCl, pH 8.0 (buffer A), using a Millipore Prep/Scale-TFF 30 kDa cartridge, and was concentrated to approximately 300 ml prior to loading onto a 5 ml Ni-NTA Superflow column (Qiagen). After extensive washing with buffer A, the column was washed sequentially with 2 column volumes of buffer A containing 30, 50, 75, 100 and 300 mM imidazole, pH 8.0. Typically the majority of the protein eluted in the 75 and 100 mM fractions. Fractions were concentrated in a Centriprep 30 (Amicon), and loaded onto a Pharmacia Superose 6 gel filtration column. The s-erbB proteins eluted as approximately 85 kDa species, and were greater than 95% pure at this stage of purification. For s-erbB1 and s-erbB4, appropriate gel-filtration fractions were pooled, diluted 1.5 fold with 50 mM MES pH 6.0, and were loaded on to an BioScale-S2 cation exchange column (BioRad), pre-equilibrated with 25 mM MES pH 6.0. Protein was eluted with a gradient in NaCl, s-erbB1 eluting at approximately 200 mM NaCl, and s-erbB4 at approximately 300 mM NaCl. Attempts to purify s-erbB2 and s-erbB3 by ion exchange led only to precipitation of the proteins at the low salt concentration required for binding to the column. Purified s-erbB proteins are buffer exchanged into 25 mM Hepes, 100 mM NaCl, pH 8.0, concentrated to between 20 and 100 μM, and stored at 4° C. Purity was checked by SDS-PAGE, and concentrations were determined by absorbance at 280 nM using calculated extinction coefficients. Molar extinction coefficients used were s-erbB1, 58900; s-erbB2, 63310; s-erbB3, 68430; s-erbb4, 73550.

Multi-angle laser light-scattering (MALLS) studies

MALLS is our primary approach to study s-erbB homo- and heterodimerization. This technique is both more sensitive and more rapid than small-angle X-ray scattering that we have used before, allowing more experiments to be done, and over a wider range of protein concentrations. A DAWN DSP Laser Photometer from Wyatt Technologies (Santa Barbara, CA) is used, which is ideally suited to these experiments (Wyatt, 1993). The DAWN contains a glass flow-cell (volume 70 μl), around which are 17 usable photodiode detectors at different angles from 15° to 160°. Scattering of light (633 nm) from a 5mW He-Ne laser is measured simultaneously at each of these angles, and normalized for variations in laser intensity as well as geometric effects (using an isotropic scatterer). The DAWN is used in micro-batch mode, samples being introduced into the flow-cell via a 0.1μm filter with a syringe pump. To avoid introduction of air-bubbles, samples are degassed under vacuum, and introduced via a low dead-volume multi-port valve, which is loaded with several samples and purged of air prior to a series of measurements. A sample of 300 μl is more than sufficient to flush and equilibrate the flow-cell for stable scattering measurements, which themselves are observed in real time. With adjustments to the gain of the detector amplifiers, scattering from sEGFR samples of less than 0.01 mg/ml (0.1 μM) to greater than 10 mg/ml (100 μM) can be measured accurately. Data are collected and analyzed using the ASTRA software supplied with the instrument. For micro-batch experiments, we inject a series of samples with fixed sEGFR concentration and increasing concentrations of EGF. Scattering data at all 17 angles are collected until the response is stable. For a region of the normalized data after equilibration for each injected sample, the software is directed to calculate a Debye plot for each time point. In the Debye plot, $R(\theta)/K^*c$ is plotted against $\sin^2(\theta/2)$, where:

- θ is the scattering angle
- $R(\theta)$ is the excess intensity (I) of scattered light at that angle ($= I(\theta)_{sample}/I(\theta)_{buffer}$)
- c is the mass concentration of the sample

K^* is a constant equal to $4\pi^2 n^2 (dn/dc)^2/\lambda_0^4 N_A$, where n = solvent refractive index, dn/dc = refractive index increment of scattering sample, λ_0 = wavelength of scattered light, N_A = Avogadro's number

Since:

$$\frac{K^* c}{R(\theta)} = \frac{1}{\overline{M}_w P(\theta)} + 2A_2 c \qquad 1$$

where A_2 is the second virial coefficient, $\overline{M}_w$ is the weight-averaged molecular mass, and:

$$P(\theta) = 1 - \frac{16\pi^2 R_G^2 \sin^2(\theta/2)}{3\lambda^2} + \ldots \qquad 2$$

then, extrapolating to zero angle $\theta = 0$ ($P(\theta) = 1$):

$$\frac{K^* c}{R(\theta)} = \frac{1}{\overline{M}_w} + 2A_2 c \qquad 3$$

By extrapolating the Debye plot to zero angle (when $R(\theta)/K^*c = \overline{M}_w$), the weight-averaged molecular mass ($\overline{M}_w$) of the molecule in the scattering sample can be measured directly if the value of the virial coefficient (A_2) is known for the protein. We have measured A_2 for s-erbB1 (and the 1:1 EGF:s-erbB1 complex) from Zimm plots, obtaining a value of 6.5×10^{-5} mol.ml.g^{-2}. Even at the highest s-erbB1 concentrations studied (3mg/ml), A_2 contributes less than 3% to the apparent weight-averaged molecular mass. For monitoring s-erbB protein dimerization, we are interested only in relative values of $\overline{M}_w$. If the concentration of receptor is fixed, errors in determination of the degree of glycosylation will not be important.

Analytical ultracentrifugation studies

Sedimentation equilibrium experiments employed the XL-A analytical ultracentrifuge (Beckman). Samples were loaded into six-channel epon charcoal-filled centerpieces, using quartz windows. Experiments were performed at 25°C using three different speeds (6,000 and 9,000, and 12,000 r.p.m.), detecting at 280 nm, with identical results. Solvent density was taken as 1.003 g/ml, and the partial specific volumes of the s-erbB proteins were approximated from their amino acid compositions and the assumption of approximately 20% carbohydrate, as 0.71 ml g^{-1} for the purposes described in this report. Experiments were performed at 5 μM or 10 μM protein. Data were fit using the Optima XL-A data analysis software (Beckman/MicroCal) to models assuming a single ideal or non-ideal species for unliganded s-erbB proteins. When ligand was added, a two-species fit was used, in which one of the species was the excess ligand, which sediments as a 6 kDa (EGF) or 8 kDa (NRG) species. Knowing the K_D values from our BIAcore studies, the amount of free ligand is also known. The molecular mass of the s-erbB species is allowed to float in these fits. Fits were judged by the occurrence of randomly distributed residuals, examples of which are shown in Fig 6. Where possible, simple interpretation of analytical ultracentrifugation experiments was made by inspection.

BIAcore studies

Experiments employed a BIAcore 2000 instrument, and were all performed in 10 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20 at 25°C. Ligands were crosslinked to the hydrogel matrix of BIAcore CM5 Biosensor chips activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*'-[3-(diethylamino)propyl]carbodiimide (EDC). EGF at 200 μg/ml in 10 mM sodium acetate, pH 4.0 was then injected at 5 μl/min for 10 minutes. Non cross-linked

EGF was removed, and unreacted sites were blocked with 1 M ethanolamine, pH 8.5. The signal contributed by immobilized EGF ranged from 150 RU to 400 RU, depending on the specific chip. For immobilization of NRG1-β1, the procedure was essentially the same, except that immobilization was performed in 10 mM sodium acetate at pH 4.8.

The purified s-erbB proteins at a series of concentrations were each flowed simultaneously over the EGF and NRG (and mock/control) surfaces at 5 μl/min for 7 minutes, by which time binding had reached a plateau in each case. The RU value corresponding to this plateau was taken as a measure of s-erbB protein binding, and was corrected for background non-specific binding and bulk refractive index effects by subtraction of data obtained in parallel using the mock-coupled hydrogel surface. RU values were then converted into percentage maximal binding. This conversion was performed separately for each surface (since levels of immobilization varied). 100% binding was defined for an NRG surface as the highest corrected signal seen with s-erbB3 and s-erbB4 (which were always the same to within 10%), and for an EGF surface the highest corrected signal seen with s-erbB1. Buffer washes between runs were sufficient to bring th eRU value back down to baseline. Data were plotted as s-erbB concentration against percent maximal binding, and fit to a simple binding equation, in ORIGIN (MicroCal) to estimate K_D.

Other methods are described in figure legends and text above.

KEY RESEARCH ACCOMPLISHMENTS

- Produced and purified milligram quantities of the four erbB receptor extracellular domains for biophysical and structural studies
- Demonstrated that EGF induces homodimerization of the erbB1 (EGF receptor) extracellular domain, but of no other s-erbB protein
- Demonstrated that NRG induces homodimerization of the erbB4 extracellular domain, but of no other s-erbB protein
- Demonstrated that the erbB3 extracellular domain binds NRG1-β1 without being induced to dimerize, and measured K_D values for ligand binding by other s-erbB proteins.
- Showed, for the first time, ligand-induced heterodimerization of isolated erbB receptor extracellular domains, but only with NRG1-β1 inducing heterodimerization of s-erbB2 s-erbB4.
- Just as importantly, we have shown that s-erbB1 does not participate in any heterodimerization reactions, contrary to the expectations in the field.
- Demonstrated in *in vitro* studies that HB-EGF is a bispecific ligand that can bind to both s-erbB1 and s-erbB4, while NRG2 is able to bind to s-erbB4 but not to s-erbB3 or other erbB receptors.
- Demonstrated that, while the ectodomains of erbB1 and erbB2 cannot be induced to dimerize *in vitro*, they do associate with one another in a heteromeric complex of some sort *in vivo*.
- Showed that EGF can activate PI 3-kinase in MCF7 cells through the membrane-anchored erbB1 extracellular domain, arguing that the membrane-tethered ectodomain dimer may be a relevant ligand for erbB3 (and possibly erbB2).
- Illustrated that the erbB1 requirements for PI 3-kinase activation by EGF and amphiregulin are different. Amphiregulin requires the full-length receptor while EGF does not.
- Proposed a model of homodimer-nucleated heterotetramerization for transmodulation of erbB receptors.

REPORTABLE OUTCOMES

1. Results presented at an invited lecture at the 1999 Gordon Conference on "Ligand Recognition and Molecular Gating" in Ventura, CA, March 7-12, 1999
   Mark Lemmon: "Ligand-Induced dimerization of erbB receptors"

2. Results presented as a poster at the Era of Hope meeting in Atlanta GA, June 8-11, 2000: "Extracellular domains are sufficient for ligand-induced homo- but not hetero-dimerization of erbB receptors"
   by: Kathryn M. Ferguson, Timothy L. Macatee and Mark A. Lemmon

3. Paper entitled: "Extracellular domains drive homo- but not hetero-dimerization of erbB receptors", by Kathryn M. Ferguson, Paul J. Darling, Mohita J. Mohan, Timothy L Macatee, and Mark A. Lemmon, published in the *EMBO Journal,* September 2000 (*EMBO J.* **19,** 4632-4643)

4. Awarded R21 grant (R21-CA87182) by the National Cancer Institute for "Structural Studies of ErbB/Her Receptor Dimerization" P.I. Mark A. Lemmon

5. Awarded R01 grant (R01-CA79992) by the National Cancer Institute for "ErbB Receptor Homo- and Hetero-Dimerization" P.I. Mark A. Lemmon

6. Manuscript submitted to *J. Biol. Chem.* Entitled "ErbB receptor transmembrane domains homodimerize in a biological membrane" by Jeannine M. Mendrola, Mitchell B, Berger, and Mark A. Lemmon.

CONCLUSIONS

Our studies alter the view of erbB receptor activation by EGF and NRG family members. ErbB1 and erbB4 are the central receptors in the system, binding to and being homodimerized by EGF and NRG respectively. Transmodulation of erbB2 by NRG, to which it does not bind directly, may involve NRG-induced formation of hetero-oligomers with erbB3 or erbB4. Formation of these hetero-oligomers can be recapitulated, at least in part, using isolated extracellular domains. So far, these findings - observed *in vitro* for the first time - are consistent with expectations from previous cellular studies.

However, a major surprise in our studies was the inability of any erbB ligand to induced formation of any heterodimer containing the extracellular domain of erbB1, in spite of the fact that heteromeric interactions (of unknown stoichiometry) between erbB1 and erbB2 *in vivo* sparked much of the debate about erbB receptor heterodimerization.

Our findings argue:

1. That erbB1 (EGFR) activation is mechanistically distinct from EGF-induced transmodulation of erbB2, erbB3, and erbB4. We hypothesize that erbB1 homodimers must associate with the receptors that they will transmodulate, by contrast with the suggestion that heterodimers form. This changes dramatically our mechanistic view of the transmodulation event..
2. That EGF and NRG may activate erbB2 through distinct mechanisms: one (NRG) through ligand-induced heterodimer formation, and the other (EGF) through an as-yet-unclear mechanism.
3. While our supposition going into this project was that quantitative differences would explain the diversity of signaling in this system, the studies to date have identified qualitative differences that are more likely to be of use in design of therapeutic strategies.
4. It is clear from studies descrived here that membrane-tethered proteins containing just the extracellular domains of erbB1 and erbB2 can form hetero-oligomers.
5. The intracellular domain of erbB1, while critical for directly erbB1-induced responses, is completely dispensable for EGF (but not ARG-induced) transmodulation of erbB3 as assessed by monitoring PI 3-kinase activty. Thus, we propose that the extracellular portion of an erbB1 dimer may actually function in effect as a ligand for erbB2 or erbB3, inducing their dimerization 'by proxy' by presenting a surface at which other receptors can self-associate. This revised hypothesis suggests new avenues to explore for possible intervention in erbB2 activation, which will be a focus of our future research.

So What ?

A major aim in breast cancer is to inactivate or otherwise remove erbB2/Neu/Her2 in the 30% or so of cases where its over-expression is seen. Herceptin has this as the basis of its efficacy. Our studies are bringing new insights into how erbB2 is regulated in cells. In particular we find that there are TWO mechanims for erbB2 transmodulation by other receptors in the erbB family. Since breast cancer cells differ in their complement of other erbB receptors, these mechanisms are likely to be of different degrees of importance in different cases (T47D cells and SKBR-3 cells will differ, for example). Understanding the mechanisms, which we are beginning to do, will allow us to begin our approaches to designing new strategies for intervention when erbB2 is inappropriately active. Knowing when the different mechanisms are most important will allow consideration of approaches that are much more selective and specific than can possibly be true with antibody-based therapies. Our studies have provided a new view of how erbB2 is regulated, now supported by work from other labs, which will allow us to follow new directions in trying to develop approaches to reverse erbB2 activation.

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APPENDICES

Bibliography of publications and meeting abstracts

Ferguson, K.M., Darling, P.J., Macatee, T.L., Mohan, M. & Lemmon, M.A. (2000) Extracellular Domains Drive Homo- but not Hetero-Dimerization of ErbB Receptors. *EMBO J.* **19,** 4632-4643.

Mendrola, J.M., Berger, M.B., and Lemmon, M.A. (2001) ErbB receptor transmembrane domains homodimerize in a biological membrane submitted to *J. Biol. Chem.*

Lemmon, M.A. (1999). Ligand-Induced dimerization of erbB receptors. Abstract for talk given at 1999 Gordon Conference on "Ligand Recognition and Molecular Gating" Ventura, CA, March 7-12, 1999.
Mark Lemmon:

Ferguson, K.M., Macatee, T.L., and Lemmon, M.A. (2000) Extracellular domains are sufficient for ligand-induced homo- but not hetero-dimerization of erbB receptors. Era of Hope meeting, Atlanta GA, June 8-11, 2000

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

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Extracellular domains drive homo- but not hetero-dimerization of erbB receptors

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Many different growth factor ligands, including epidermal growth factor (EGF) and the neuregulins (NRGs), regulate members of the erbB/HER family of receptor tyrosine kinases. These growth factors induce erbB receptor oligomerization, and their biological specificity is thought to be defined by the combination of homo- and hetero-oligomers that they stabilize upon binding. One model proposed for ligand-induced erbB receptor hetero-oligomerization involves simple heterodimerization; another suggests that higher order hetero-oligomers are 'nucleated' by ligand-induced homodimers. To distinguish between these possibilities, we compared the abilities of EGF and NRG1-β1 to induce homo- and hetero-oligomerization of purified erbB receptor extracellular domains. EGF and NRG1-β1 induced efficient homo-oligomerization of the erbB1 and erbB4 extracellular domains, respectively. In contrast, ligand-induced erbB receptor extracellular domain hetero-oligomers did not form (except for s-erbB2–s-erbB4 hetero-oligomers). Our findings argue that erbB receptor extracellular domains do not recapitulate most heteromeric interactions of the erbB receptors, yet reproduce their ligand-induced homo-oligomerization properties very well. This suggests that mechanisms for homo- and hetero-oligomerization of erbB receptors are different, and contradicts the simple heterodimerization hypothesis prevailing in the literature.

Keywords: dimerization/growth factor/scattering/signaling/tyrosine kinase

Introduction

The epidermal growth factor (EGF) receptor is the prototype of the erbB family of receptor tyrosine kinases (RTKs) that also includes erbB2 (HER-2 or Neu), erbB3 (HER-3) and erbB4 (HER-4) (Carraway and Cantley, 1994; Alroy and Yarden, 1997; Riese and Stern, 1998). Each erbB receptor contains an extracellular ligand-binding domain of 600–630 amino acids, a single transmembrane α-helix, plus an intracellular domain of ~600 amino acids that includes the tyrosine kinase and regulatory sequences (Schlessinger and Ullrich, 1992). It was established more than a decade ago for the EGF receptor (erbB1) that growth factor-induced receptor oligomerization is critical for transmembrane signaling (Schechter *et al.*, 1979; Schlessinger, 1979; Yarden and Schlessinger, 1987a,b). It is now generally accepted that the cytoplasmic tyrosine kinases of two (or more) RTKs in a growth factor-induced dimer (or larger oligomer) mutually activate one another through transphosphorylation (Honegger *et al.*, 1990; Lemmon and Schlessinger, 1994; Heldin, 1995; Hubbard *et al.*, 1998). Several downstream signaling molecules are then recruited to the phosphorylated receptor, specified by its complement of regulatory tyrosine phosphorylation sites (Songyang *et al.*, 1993; Schlessinger, 1994).

Many cells co-express multiple members of the erbB receptor family, which can form both homo- and hetero-oligomers upon stimulation with growth factor ligands (Heldin, 1995). Oligomers containing almost every possible pairwise combination of erbB receptors have now been reported (reviewed by Carraway and Cantley, 1994; Alroy and Yarden, 1997; Riese and Stern, 1998). The earliest evidence for hetero-oligomerization of erbB receptors came from the finding that erbB2 can be activated by EGF, despite the fact that it does not bind directly to this ligand. EGF is only able to activate erbB2 when erbB1 is also present in the same cell, suggesting 'transmodulation' of erbB2 as a result of its EGF-induced hetero-oligomerization with erbB1 (King *et al.*, 1988; Stern and Kamps, 1988; Goldman *et al.*, 1990; Wada *et al.*, 1990; Spivak-Kroizman *et al.*, 1992).

There are >10 distinct ligands that activate erbB receptors. Three of these have been classified as 'EGF agonists' (Riese and Stern, 1998), since they bind directly to only erbB1 [EGF, transforming growth factor-α (TGF-α) and amphiregulin]. Four (or more) of the ligands are specific for erbB3 and/or erbB4 (the neuregulins; NRGs), while a further three have been classified as 'bispecific' and bind directly to both erbB1 and erbB4 [betacellulin, epiregulin and possibly heparin-binding EGF-like factor (HB-EGF)] (Riese and Stern, 1998; Harari *et al.*, 1999; J.T.Jones *et al.*, 1999, and references therein). The EGF agonists activate erbB1 when it is expressed alone, but also transmodulate erbB2, erbB3 and erbB4 in an erbB1-dependent manner. Similarly, the NRGs activate erbB4 directly, but can also transactivate erbB1 or erbB2 when erbB4 or erbB3 are also present (Riese *et al.*, 1995). Finally, the bispecific ligands appear to activate erbB1 and erbB4 when either is expressed alone, and to transmodulate erbB2 and erbB3 via these receptors (reviewed by Alroy and Yarden, 1997; Riese and Stern, 1998). ErbB2, which is of particular medical interest as a target of breast cancer therapies (Sliwkowski *et al.*, 1999), has no known ligand and can only be activated *in trans* by ligands in these three classes. In fact, erbB2 is considered to be a preferred hetero-oligomerization partner for all of the

other erbB receptors (Karunagaran *et al.*, 1996; Graus-Porta *et al.*, 1997).

Several possible mechanisms for erbB receptor transmodulation have been considered. In the simplest and most often discussed, transmodulation is proposed to result from ligand-induced receptor heterodimerization (Alroy and Yarden, 1997; Burden and Yarden, 1997; Riese and Stern, 1998). According to this mechanism, a ligand stimulates two receptors to come together. If the two receptors are identical, this is homodimerization; if not, it is heterodimerization. Either way, the two receptors in the dimer become activated by transphosphorylation, and transmembrane signaling is achieved. Several studies argue that erbB receptor extracellular domains are sufficient for their hetero-oligomerization (Qian *et al.*, 1994), and combinatorial receptor (homo- or hetero-) dimerization could be driven by simultaneous binding of bivalent erbB ligands to the extracellular domains of two receptor molecules (Lemmon *et al.*, 1997; Tzahar *et al.*, 1997). Different bivalent ligands could stabilize distinct receptor homo- and/or heterodimers depending on the combination of binding sites that they contain.

An alternative view is that growth factors such as EGF induce only homodimerization of the erbB receptors to which they bind directly. The resulting receptor homodimers may then activate *in trans* the erbB receptors to which the ligand does not bind, through quite different mechanisms. For example, transmodulation of erbB2 by EGF could simply involve phosphorylation of erbB2 as a substrate for the activated EGF receptor. Another possibility (Huang *et al.*, 1998) is that EGF-induced erbB1 homodimers could provide an interface at which dimerization of erbB2 is promoted. ErbB2 could thus become activated by 'proxy' in the context of an $(erbB1)_2(erbB2)_2$ heterotetramer. A model of this sort could explain the surprising observation that a kinase-negative form of erbB1 can transmodulate erbB2 upon EGF binding (Wright *et al.*, 1995).

In order to determine whether erbB receptor homo- and hetero-oligomerization occur through similar mechanisms, we have studied the effects of ligand binding on the assembly of isolated erbB receptor extracellular domains. We reported previously that the isolated erbB1 extracellular domain (s-erbB1) homodimerizes quantitatively upon binding to EGF or TGF-α (Lemmon *et al.*, 1997). Here, we show that NRG1-β1 can also induce homo-oligomerization of the erbB4 extracellular domain. In contrast, ligand-induced hetero-oligomerization appears to be the exception rather than the rule for erbB receptor extracellular domains. While NRG1-β1 can induce the formation of hetero-oligomers that contain the erbB2 and erbB4 extracellular domains, no evidence could be obtained for EGF-induced formation of any extracellular domain hetero-oligomer. These findings indicate that erbB receptors form homo- and hetero-oligomers through quite different mechanisms, and that transmodulation of erbB receptors is most probably nucleated by a ligand-induced erbB1 or erbB4 homodimer.

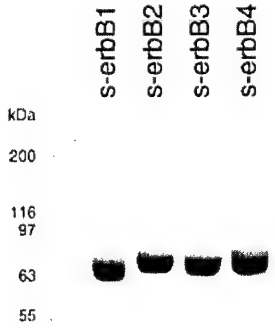

Fig. 1. SDS–PAGE (7.5%) of the purified s-erbB proteins used for analysis of ligand-induced homo- and hetero-oligomerization. Purified protein (15 μl) was loaded at a concentration of 1 mg/ml, and the gel was stained with Coomassie Blue. Molecular mass standards were loaded in the left-most lane, and are marked.

Results

High-affinity ligand binding by recombinant s-erbB proteins

To investigate the ligand binding and dimerization properties of soluble erbB receptor extracellular domains (s-erbBs), we first established methods for their production in milligram quantities by secretion from baculovirus-infected Sf9 cells (Figure 1). Using surface plasmon resonance (BIAcore), we next measured binding of each purified s-erbB protein to both EGF and NRG1-β1 that were immobilized on BIAcore CM-5 sensor chips. The s-erbB proteins were passed across these surfaces at a variety of concentrations, and the maximum response observed was plotted against s-erbB concentration to generate the binding curves shown in Figure 2A. As anticipated, s-erbB1 bound strongly to the EGF-derivatized sensor surface (K_D = 118 nM), but not to surfaces carrying NRG1-β1 or to surfaces with no ligand. Both s-erbB3 and s-erbB4 bound strongly to the NRG1-β1 surface (K_D values of 249 and 179 nM, respectively; see Table I), but not to the EGF-derivatized surface. In contrast, s-erbB2 did not bind to any of the surfaces tested (Figure 2A). We repeated these experiments using 1:1 mixtures of different s-erbB proteins (e.g. s-erbB2 plus s-erbB3 or s-erbB4) to determine whether free s-erbB proteins might hetero-oligomerize, leading to significant alterations in their apparent ligand-binding affinities. In these studies, mixing s-erbB proteins had no detectable influence on their ligand-binding properties (not shown), arguing that s-erbB hetero-oligomers (if they form) do not bind the immobilized ligands with a significantly higher affinity than single s-erbB species.

s-erbB1 and s-erbB4 homo-oligomerize upon ligand binding, while s-erbB3 does not

To analyze ligand-induced dimerization of s-erbB proteins, we employed multi-angle laser light scattering (MALLS) and sedimentation equilibrium analytical ultracentrifugation, both of which give information on molecular mass changes that is independent of molecular shape (Cantor and Schimmel, 1980).

Multi-angle laser light-scattering studies. MALLS allows the weight-averaged molecular mass ($\bar{M}_w$) of proteins in solution to be measured rapidly over a wide

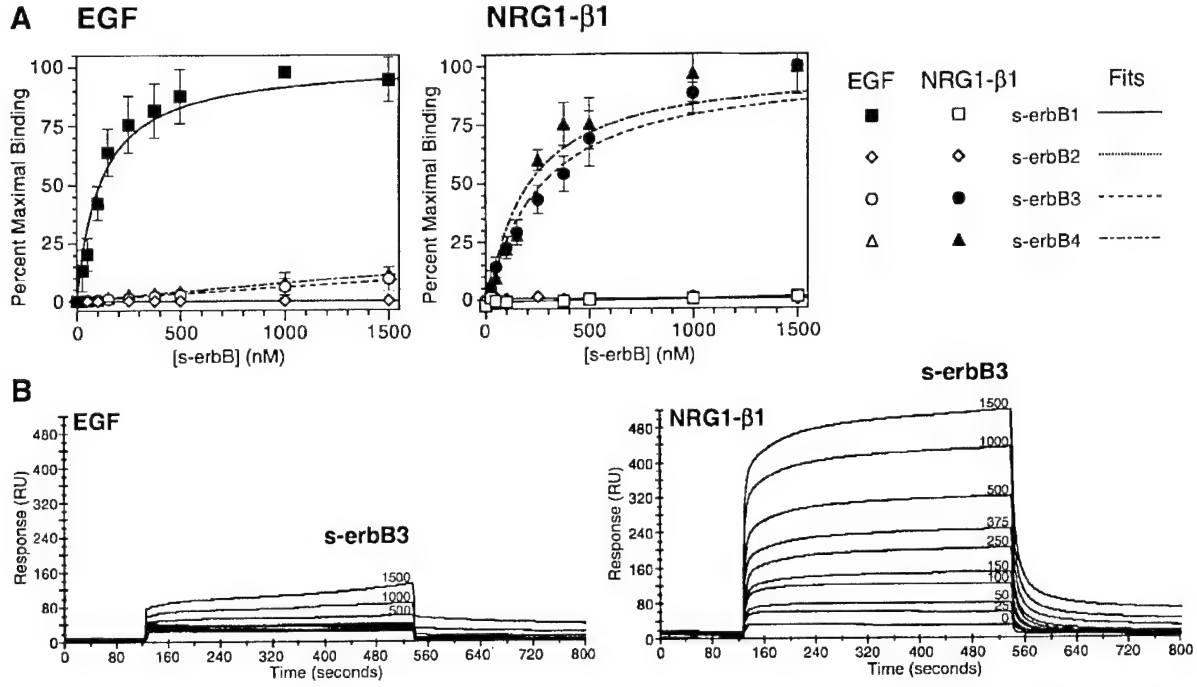

Fig. 2. (**A**) Data for binding of s-erbB1, s-erbB2, s-erbB3 and s-erbB4 to EGF (left) and NRG1-β1 (right), immobilized on a BIAcore sensor chip. Best fits to the data, assuming a simple association model, are shown. Errors are standard deviations from the mean of at least four independent determinations at each point. K_D values represented by the best fits are listed in Table I. (**B**) Representative raw BIAcore data for s-erbB3 flowed in parallel over a biosensor chip derivatized with EGF (left) and NRG1-β1 (right) at a series of different concentrations (marked on each curve in nM).

Table I. Ligand binding by s-erbB proteins

Ligand	K_D (nM)			
	s-erbB1	s-erbB2	s-erbB3	s-erbB4
EGF	118 ± 41	none	$>10^4$	$>10^4$
NRG-β1	$>10^5$	none	249 ± 80	179 ± 10

K_D values measured using BIAcore for binding of s-erbB proteins to immobilized EGF and NRG1-β1. Means of at least four independent determinations are quoted alongside their standard deviations.

range of protein concentrations (see Materials and methods). MALLS measurements gave an $\bar{M}_w$ value of 77 ± 8 kDa for purified s-erbB1 alone. When EGF is titrated effectively into an s-erbB1 solution (with fixed s-erbB1 concentration), $\bar{M}_w$ increases in a linear fashion until one molar equivalent of EGF has been added to s-erbB1 (Figure 3A). At this point, $\bar{M}_w$ is 2.2-fold higher than that measured for s-erbB1 alone, suggesting EGF-induced formation of a dimeric complex containing two EGF molecules plus two molecules of s-erbB1, as we have observed with other methods (Lemmon *et al.*, 1997). No further increase in $\bar{M}_w$ is seen when EGF is added in excess, arguing that higher order oligomers of s-erbB1 do not form. The curve through the data in Figure 3A represents the results expected if EGF binds to monomeric s-erbB1 with a K_D = 118 nM (Table I), and the resulting 1:1 (EGF:s-erbB1) complex dimerizes completely. The K_D for this dimerization event (which is complete at 4 μM s-erbB1) appears to be <0.1 μM, based on additional MALLS studies at low concentration and gel filtration experiments (not shown).

Similar MALLS studies of s-erbB4 gave a $\bar{M}_w$ of 82 ± 6 kDa that increased by a factor of >2 as NRG1-β1 was added (Figure 3B). In this case, the maximum $\bar{M}_w$ value was not reached until more than two equivalents of NRG1-β1 had been added. Furthermore, the final $\bar{M}_w$ value (~235 kDa) was higher than expected for a dimeric s-erbB4–NRG1-β1 complex. These data therefore suggest that NRG1-β1 is able to induce formation of s-erbB4 oligomers that are larger than dimers. Without more detailed analysis at significantly higher protein concentrations and at larger excesses of ligand, we cannot determine the maximum oligomeric state. However, an increase of nearly 3-fold in $\bar{M}_w$ (at an NRG1-β1:s-erbB4 ratio of 3:1) is equally consistent with the formation of s-erbB4 trimers and with the formation of a mixture that contains 50% of the s-erbB4 as dimers plus 50% as tetramers.

We also used MALLS to analyze the ability of NRG1-β1 to induce s-erbB3 oligomerization. As shown by a single data point in Figure 3B (and confirmed in centrifugation studies described below), addition of a 2-fold excess of NRG1-β1 did not increase the $\bar{M}_w$ measured for s-erbB3 above that measured for s-erbB3 alone (90 ± 4 kDa). This finding is consistent with a previous report (Horan *et al.*, 1995), and does not reflect a lack of NRG1-β1 binding by s-erbB3 (see Figure 2B and Table I). Addition of neither EGF (Figure 3A) nor NRG1-β1 (not shown) altered the value measured for s-erbB2 (78 ± 10 kDa), as was expected since neither ligand binds to this protein (Figure 2A).

Analytical ultracentrifugation. Sedimentation equilibrium experiments gave the same results for ligand-induced

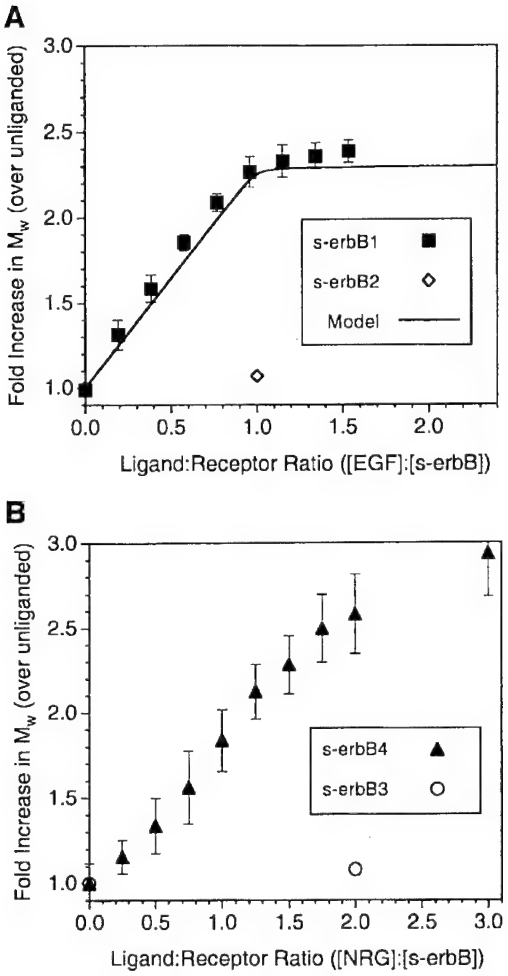

Fig. 3. MALLS studies of EGF-induced homodimerization of s-erbB1- (**A**) and NRG1-β1-induced homo-oligomerization of s-erbB4 (**B**). The weight-averaged molecular mass ($\bar{M}_w$) of s-erbB1:EGF mixtures (relative to for s-erbB1 alone), as determined by MALLS (see Materials and methods), is plotted against the EGF:s-erbB1 ratio in the mixture. Quantitative EGF-induced s-erbB1 homodimerization is shown (filled squares). The solid line represents the expected results for a model in which EGF binds s-erbB1 with a K_D of 118 nM, and the resulting 1:1 complex dimerizes with a K_D of 100 nM (see text). The single open diamond in (A) shows one point for a similar experiment with s-erbB2, demonstrating that s-erbB2 does not dimerize when EGF is added (see also Figure 4B). In (B), the same experiment is shown for NRG1-β1 binding to s-erbB4 (filled triangles), which it causes to oligomerize. Also in (B), a single point (open circle) shows the failure of NRG1-β1 to induce s-erbB3 homo-oligomerization. Error bars correspond to the standard deviations for the mean of three or more experiments. The concentration of s-erbB protein was 4 μM in each experiment.

s-erbB protein homo-oligomerization. Figure 4 shows typical data from sedimentation equilibrium experiments (at 6000 r.p.m.) in which 5 μM samples of each s-erbB protein were centrifuged both with (filled symbols) and without (open symbols) a 2-fold molar excess of the most relevant growth factor ligand. Data obtained with the ligand-free s-erbB proteins can be fit, using a model that assumes a single non-ideal species, to give molecular mass estimates of 81 ± 1 kDa (s-erbB1), 80 ± 3 kDa (s-erbB2), 82 ± 7 kDa (s-erbB3) and 81 ± 3 kDa (s-erbB4). The residuals for these fits, plotted above the data in Figure 4, are both small and random, indicating good fits. When EGF is added to s-erbB1 (Figure 4A), or NRG1-β1 is added to s-erbB4 (Figure 4D), the radial distribution plots suggest a substantial increase in molecular mass (with material accumulating at higher radii). Since the molecular masses of EGF and NRG1-β1 are only 6 and 8 kDa, respectively (Lemmon *et al.*, 1997; data not shown), and those of s-erbB1 and s-erbB4 are ~80 kDa, this effect can only be explained by homo-oligomerization of the s-erbB proteins upon addition of the relevant growth factor. The data for s-erbB:ligand mixtures can be fit using a model that assumes two ideal species: the ligand–receptor complex and excess ligand. Using this model, the masses of s-erbB1–EGF and s-erbB4–NRG1-β1 complexes are estimated as 159 ± 10 kDa and 146 ± 18 kDa, respectively (residuals for these fits are shown in Figure 4A and D), consistent with the ligand-induced oligomerization of these extracellular domains seen by MALLS. In other sedimentation experiments (not shown), TGF-α and HB-EGF were also found to induce formation of s-erbB1 homo-oligomers (assumed dimers). As with MALLS, sedimentation equilibrium studies of s-erbB4:NRG1-β1 mixtures at higher s-erbB4 concentrations and larger ligand excesses (not shown) suggested that NRG1-β1 induces formation of s-erbB4 oligomers larger than dimers. However, we have not yet been able to determine whether these are trimers or mixtures of different oligomers.

In contrast to the findings for s-erbB1 and s-erbB4, no indication of ligand-induced oligomerization was seen when EGF was added to s-erbB2 (Figure 4B), or when NRG1-β1 was added to either s-erbB3 (Figure 4C) or s-erbB2 (see below). The data for the s-erbB2:EGF mixture were best fit as a combination of free EGF and free s-erbB2 (82 ± 12 kDa), and those for the s-erbB3:NRG1-β1 mixture fit best as free NRG1-β1 (8 kDa) plus a 1:1 s-erbB3–NRG1-β1 complex of 83 ± 17 kDa.

ErbB1 and erbB2 extracellular domains do not heterodimerize upon EGF binding

Having confirmed that EGF induces s-erbB1 homodimerization, and that NRG1-β1 induces s-erbB4 homo-oligomerization, we next investigated the ability of erbB ligands to induce heterodimerization of erbB receptor extracellular domains. As described in the Introduction, the most well-studied example of erbB receptor transmodulation involves erbB1 and erbB2. Since EGF induces complete homodimerization of s-erbB1, we expected from the simple heterodimerization model for erbB receptor transmodulation that EGF should also induce the formation of s-erbB1–s-erbB2 heterodimers.

Contrary to these expectations, heterodimer formation could not be observed in MALLS studies when EGF was added to a 1:1 mixture of s-erbB1 and s-erbB2. Instead, EGF induced homodimerization of s-erbB1 in the mixture, while s-erbB2 remained monomeric. As shown in Figure 5A, titration of EGF into a solution containing 4 μM (crossed-squares) or 8 μM (filled squares) s-erbB1 alone caused complete dimerization. $\bar{M}_w$ reached a maximum value (~2-fold) after addition of EGF to ~4 and 8 μM, respectively, as expected for the formation of a 2:2 EGF:s-erbB1 dimer. If EGF-induced heterodimeriza-

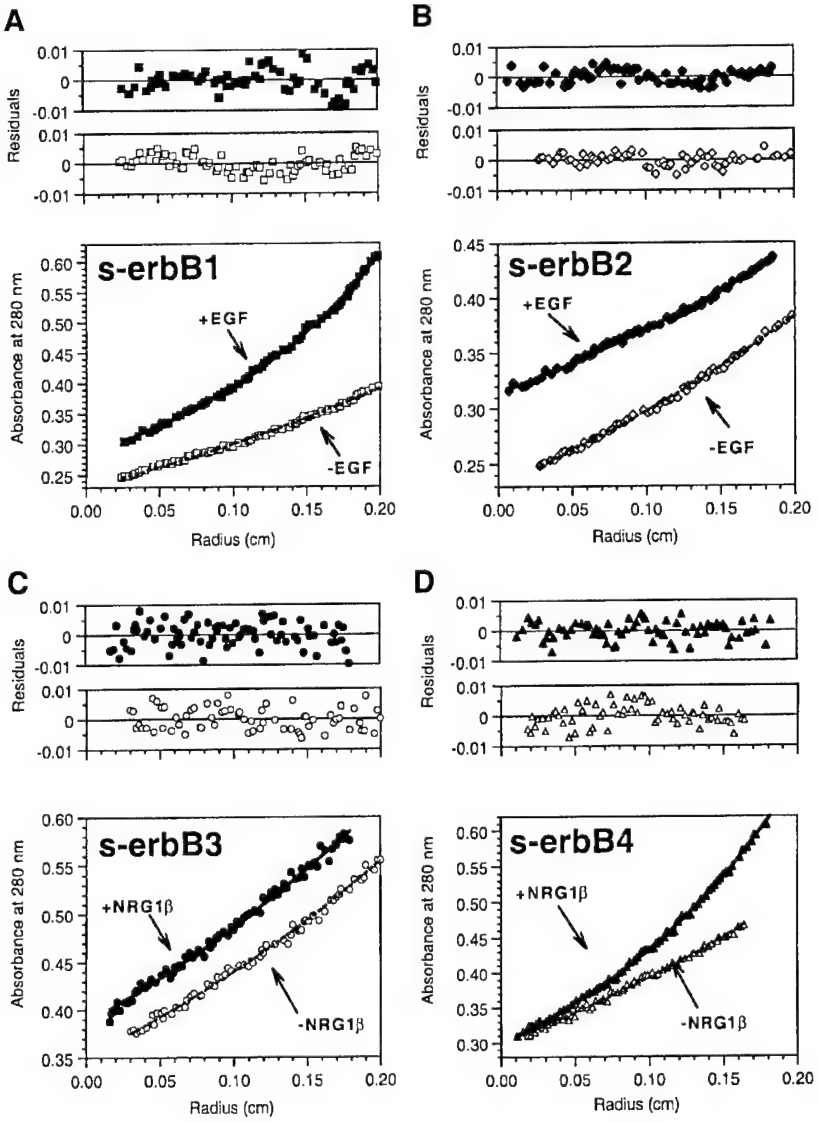

Fig. 4. Representative sedimentation equilibrium analytical ultracentrifugation data for analysis of s-erbB homo-oligomerization induced by EGF (**A** and **B**) or NRG1-β1 (**C** and **D**). In each case, open symbols represent s-erbB protein without added ligand, which is fit as a single non-ideal species. Filled symbols represent samples to which a 2-fold molar excess of the noted ligand has been added. As discussed in the text, fits to these data are with two ideal species (complex plus excess free ligand)—fixing the mass of the ligand and floating the mass of the complex. Purified s-erbB protein was used at 5 μM for each sample. All experiments shown were performed at 6000 r.p.m. Repeats at 9000 and 12 000 r.p.m. gave the same results. Residuals for the fits described above are shown, and are seen to be both small and random, indicative of a good fit. EGF induced homo-oligomerization of s-erbB1 only, while NRG1-β1 induced homo-oligomerization of s-erbB4 only. Radius is plotted as $(r - r_o)$, where r is the radial position in the sample, and r_o the radial position of the meniscus.

tion of s-erbB1 with s-erbB2 were similarly strong, MALLS data for a 1:1 s-erbB1:s-erbB2 mixture (8 μM total receptor) should resemble that seen for 8 μM s-erbB1 alone. However, EGF addition to such a 1:1 mixture (diamonds in Figure 5A) induced a maximum $\bar{M}_w$ increase of only 1.6-fold, and this maximum was reached at 4 μM, not 8 μM, total EGF. Homodimerization of just s-erbB1 (at 4 μM) in this mixture would be maximal at 4 μM EGF according to the data in Figure 3A. Furthermore, a 1.6-fold increase in $\bar{M}_w$ is exactly what is expected if s-erbB1 homodimerizes (yielding 174 kDa s-erbB1 dimers at 2 μM) while s-erbB2 remains monomeric (80 kDa s-erbB2 monomers at 4 μM). Therefore, EGF does not induce heterodimerization of s-erbB1 with s-erbB2—or at least the K_D for this heterodimerization event is sufficiently weak to be undetectable under these conditions (where s-erbB1 homodimerization is complete).

Sedimentation equilibrium experiments also argue strongly against EGF-induced s-erbB1–s-erbB2 heterodimerization. For a set of experiments performed at 6000 r.p.m., the natural logarithm of absorbance at 290 nm (proportional to protein concentration) is plotted in Figure 5B against $(r^2 - r_o^2)/2$, where r is the radial position in the sample, and r_o the radial position of the meniscus. For an ideal single species, this plot is linear and the gradient of the line $[M\omega^2(1 - \bar{V}_2\rho)/RT]$ is proportional to the molecular mass (M) of the ideal species (Cantor and Schimmel, 1980). The data for s-erbB1 or s-erbB2 alone fit

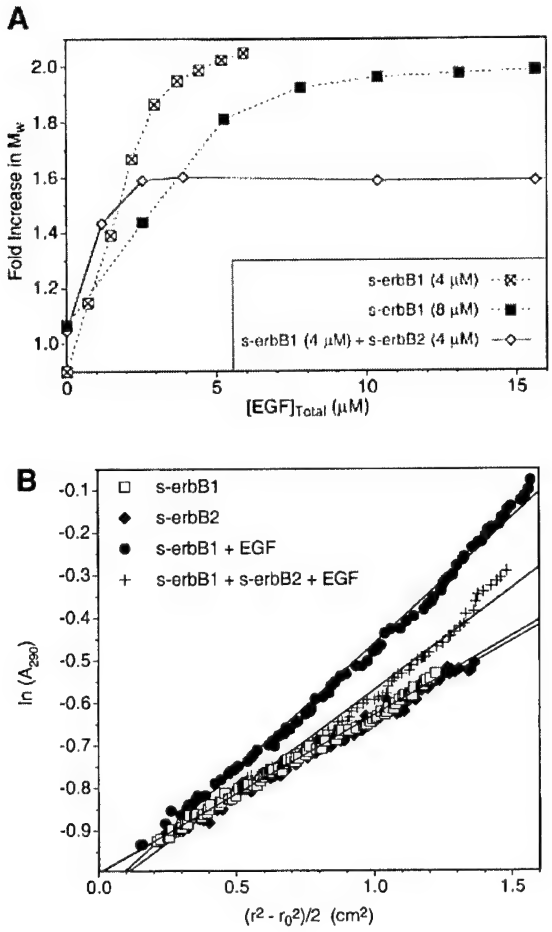

Fig. 5. (**A**) A MALLS experiment demonstrating that, while EGF induces complete homo-dimerization of s-erbB1 at 4 μM (crossed squares) or 8 μM (filled squares), it does not induce the formation of heterodimers between s-erbB2 and s-erbB1 (open diamonds). The experiment was performed as described for Figure 3. With 4 μM s-erbB1, complete dimerization is seen after addition of 4 μM EGF (note that the horizontal axis here is EGF concentration, and not ligand:receptor ratio). With 8 μM s-erbB1, addition of 8 μM EGF is required for complete dimerization. When the 1:1 s-erbB1:s-erbB2 mixture is studied, with a total s-erbB protein concentration of 8 μM, only 4 μM EGF is required for maximal dimerization, and the maximum fold increase in M_w is consistent only with a mixture of s-erbB1 homodimers and s-erbB2 monomers. Lines are drawn to guide the eye, and do not represent fits to the data. (**B**) Plots of the natural logarithm of absorbance at 290 nm (monitoring protein concentration) against a function of the radius squared $(r^2 - r_0^2)/2$ (see text for explanation) for sedimentation equilibrium analytical ultracentrifugation data obtained at 6000 r.p.m. with s-erbB1 and s-erbB2. For an ideal single species, this representation of the data should appear as a straight line with a gradient proportional to the molecular mass (see text). When analyzed alone, both s-erbB1 (open squares) and s-erbB2 (filled diamonds) yield good straight lines, with gradients proportional to their monomeric molecular masses (see also fits in Figure 4). Each sample contained a total s-erbB concentration of 10 μM. The increase in gradient for the s-erbB1/s-erbB2/EGF mixture (crosses) is consistent with the formation of s-erbB1 homodimers only.

well to a straight line with a gradient that suggests a molecular mass of ~80 kDa in each case. When two molar equivalents of EGF were added to s-erbB1, the gradient of the best straight line (Figure 5B, filled squares) was increased substantially over that for s-erbB1 alone, because of EGF-induced s-erbB1 homodimerization. When the same excess of EGF was added to a 1:1 s-erbB1:s-erbB2 mixture (two EGFs added per s-erbB molecule), the data fit less well to a straight line (indicating multiple species), and the gradient of the best line was increased only slightly over that for s-erbB1 or s-erbB2 alone. Similar experiments at substantially higher receptor concentrations also failed to provide evidence for erbB1–erbB2 hetero-oligomerization. Thus, as seen with MALLS, analytical ultracentrifugation studies suggest that EGF induces homodimerization of s-erbB1 in a s-erbB1:s-erbB2 mixture, while s-erbB2 remains monomeric.

These biophysical studies show that the isolated extracellular domains of erbB1 and erbB2 do not associate with one another in a heterodimer (or any other oligomer) upon EGF addition, whereas s-erbB1 homodimerizes efficiently upon EGF binding (Figures 3–5) and EGF-dependent co-immunoprecipitation of intact erbB1 and erbB2 has been reported by many groups. In studies not shown, we attempted to detect s-erbB1–s-erbB2 interactions using chemical cross-linking and co-immunoprecipitation approaches, and obtained only negative results (although s-erbB1 homodimers could be seen readily by chemical cross-linking). We therefore suggest that, while the extracellular domain is sufficient for EGF-induced homodimerization of erbB1, extracellular domains are not capable of driving receptor hetero-oligomerization. Before concluding this, however, an important caveat must be considered. Since erbB2 has no known ligand, we cannot validate the functional integrity of Sf9 cell-derived s-erbB2 by virtue of its ligand binding, as was possible with s-erbB1, s-erbB3 and s-erbB4 (Figure 2). However, we believe that s-erbB2 is functional, since it appears to form NRG1-induced hetero-oligomers with s-erbB4 (see below).

ErbB1 and erbB4 extracellular domains do not hetero-oligomerize upon EGF or NRG1-β1 binding

Evidence for hetero-oligomerization (or transmodulation) of erbB1 and erbB4 upon treatment of cells with either EGF or NRG has been reported by several groups (Riese *et al.*, 1995, 1996; Cohen *et al.*, 1996; Zhang *et al.*, 1996; F.E.Jones *et al.*, 1999). We therefore used analytical ultracentrifugation to investigate whether EGF and NRG1-β1 induce s-erbB1–s-erbB4 heterodimerization. Since we know that s-erbB1 and s-erbB4 are both competent to homo-oligomerize upon binding of EGF and NRG1-β1, respectively, we can be confident that these proteins are functionally active.

A series of sedimentation equilibrium experiments was performed with 1:1 mixtures of s-erbB1 and s-erbB4, with the same total receptor concentration (8 μM) in each case (Figure 6). With no ligand added, the gradient of the straight line through the data gives an average monomeric molecular mass of ~80 kDa. Addition of EGF to a concentration twice that of total receptor (i.e. two EGF molecules per s-erbB1 molecule plus two EGF molecules per one s-erbB4) increases the gradient of the straight line only slightly (circles in Figure 6), suggesting that some oligomerization is induced. Addition of only NRG1-β1 to the same final concentration gives a similar result (triangles in Figure 6). Since ligand is not limiting in

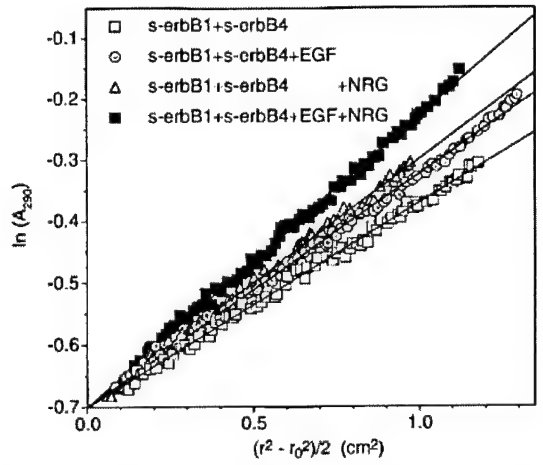

Fig. 6. Analytical ultracentrifugation data, presented as ln(Abs) against $(r^2 - r_o^2)/2$ plots, to study s-erbB1–s-erbB4 hetero-oligomerization. The s-erbB1:s-erbB4 mixture (8 μM total [s-erbB]) without ligand gives a straight line with the gradient expected for monomeric protein (open squares). Addition of EGF alone (16 μM) or NRG alone (16 μM) results in a modest increase in molecular mass that is consistent with homo-oligomerization of one species only (gray circles and triangles, respectively). Addition of both EGF and NRG (8 μM each) results in a substantially larger increase in the gradient (black squares), indicating that both species homo-oligomerize independently, and do not form hetero-oligomers (see text for explanation).

either of these cases, we hypothesized that these small increases in gradient result from homo-oligomerization of just s-erbB1 when EGF is added, and of just s-erbB4 when NRG1-β1 is added. If this is true, an identical sample containing the same total ligand concentration, but as a 1:1 mixture of EGF and NRG1-β1 (i.e. with two EGF molecules per s-erbB1 molecule plus two NRG1-β1 molecules per one s-erbB4), should give a substantially steeper gradient by inducing independent homo-oligomerization of both s-erbB1 and s-erbB4. Indeed, the steepest line in Figure 6 (filled squares) shows this to be the case, arguing that s-erbB1 and s-erbB4 do not form hetero-oligomers under these conditions with either EGF or NRG1-β1.

Evidence for NRG1-β1-induced hetero-oligomerization of s-erbB4 and s-erbB2

The experiments described above show that EGF does not induce hetero-oligomerization of s-erbB1 with s-erbB2 or s-erbB4. Other experiments showed that EGF does not induce the formation of s-erbB1–s-erbB3 or s-erbB2–s-erbB3 hetero-oligomers, and that NRG1-β1 does not drive the interaction of s-erbB1 with s-erbB3 (not shown). Therefore, although EGF-induced s-erbB1 homodimerization is highly efficient, s-erbB1 does not participate in formation of any s-erbB hetero-oligomer. Furthermore, EGF cannot induce the formation of any s-erbB hetero-oligomer. To compare these properties of EGF with those of NRG1-β1, we next tested the ability of NRG1-β1 to induce formation of a series of s-erbB dimers (Figure 7). Using linearized sedimentation equilibrium data as a qualitative guide, Figure 7A, B and C shows that NRG1-β1 induces homo-oligomerization of s-erbB4 (see also Figures 3B and 4D), but not of s-erbB2 or s-erbB3. The data for s-erbB4 homo-oligomerization (from Figure 7A) are superimposed upon all other graphs in Figure 7 to aid comparison. NRG1-β1 addition to an s-erbB2:s-erbB3 mixture caused a slight increase in the gradient of the best straight line through the data (Figure 7D), suggesting that there may be very weak hetero-oligomerization of these proteins (although much weaker than s-erbB4 homo-oligomerization). The data obtained with a s-erbB3:s-erbB4 mixture (Figure 7E) are most consistent with NRG1-β1 inducing independent homo-oligomerization of s-erbB4, with no effect on s-erbB3 (as seen for NRG1-β1 addition to a s-erbB1/s-erbB4 mixture) and therefore do not suggest a hetero-oligomerization event.

Figure 7F shows the most interesting of these results, and represents the only data in this study that argue for ligand-induced s-erbB hetero-oligomerization. In the absence of NRG1-β1, sedimentation of the s-erbB2:s-erbB4 mixture is indistinguishable from that of unliganded s-erbB4. When NRG1-β1 is added, sedimentation of the s-erbB2:s-erbB4 mixture is almost identical to that seen with s-erbB4 alone (at the same total s-erbB concentration). This argues that NRG1-β1 addition induces the same increase in average molecular mass regardless of whether all of the s-erbB molecules in the sample are s-erbB4, or half of them are s-erbB2. There are two possible explanations for this. One is that NRG1-β1 can induce homo-oligomerization of s-erbB2 (as well as that of s-erbB4), which Figure 7B shows to be false. The other explanation is that hetero-oligomers containing s-erbB2 plus s-erbB4 are induced by NRG1-β1 with an efficiency similar to s-erbB4 homo-oligomerization. Independent MALLS studies (not shown) also showed that the addition of 1.5-fold molar excess of NRG1-β1 induces the same increase in weight-averaged molecular mass for a 1:1 s-erbB2:s-erbB4 mixture as it does for a solution of s-erbB4 alone, again suggesting NRG1-β1-induced s-erbB2–s-erbB4 hetero-oligomerization.

Discussion

Using analytical ultracentrifugation and MALLS, we have shown that EGF induces efficient homodimerization of the EGF receptor extracellular domain (s-erbB1), but does not induce formation of any detectable hetero-oligomers (or other homo-oligomers) of erbB receptor extracellular domains. Similar studies with NRG1-β1 showed that this ligand induces efficient homo-oligomerization of the erbB4 extracellular domain (s-erbB4), but no other s-erbB homo-oligomers. The s-erbB4 oligomers induced by NRG1-β1 appear to be larger than dimers, although we have not yet established their maximum size. As well as inducing s-erbB4 homo-oligomerization, NRG1-β1 appears to stabilize the formation of hetero-oligomers containing both s-erbB4 and s-erbB2. The qualitative results of our studies are summarized in Table II.

Comparisons with previous studies

The K_D value reported in Table I for EGF binding by s-erbB1 (118 nM) is comparable with values previously reported (100–500 nM) for EGF binding by monomeric s-erbB1 (Greenfield *et al.*, 1989; Günther *et al.*, 1990; Hurwitz *et al.*, 1991; Lax *et al.*, 1991; Zhou *et al.*, 1993;

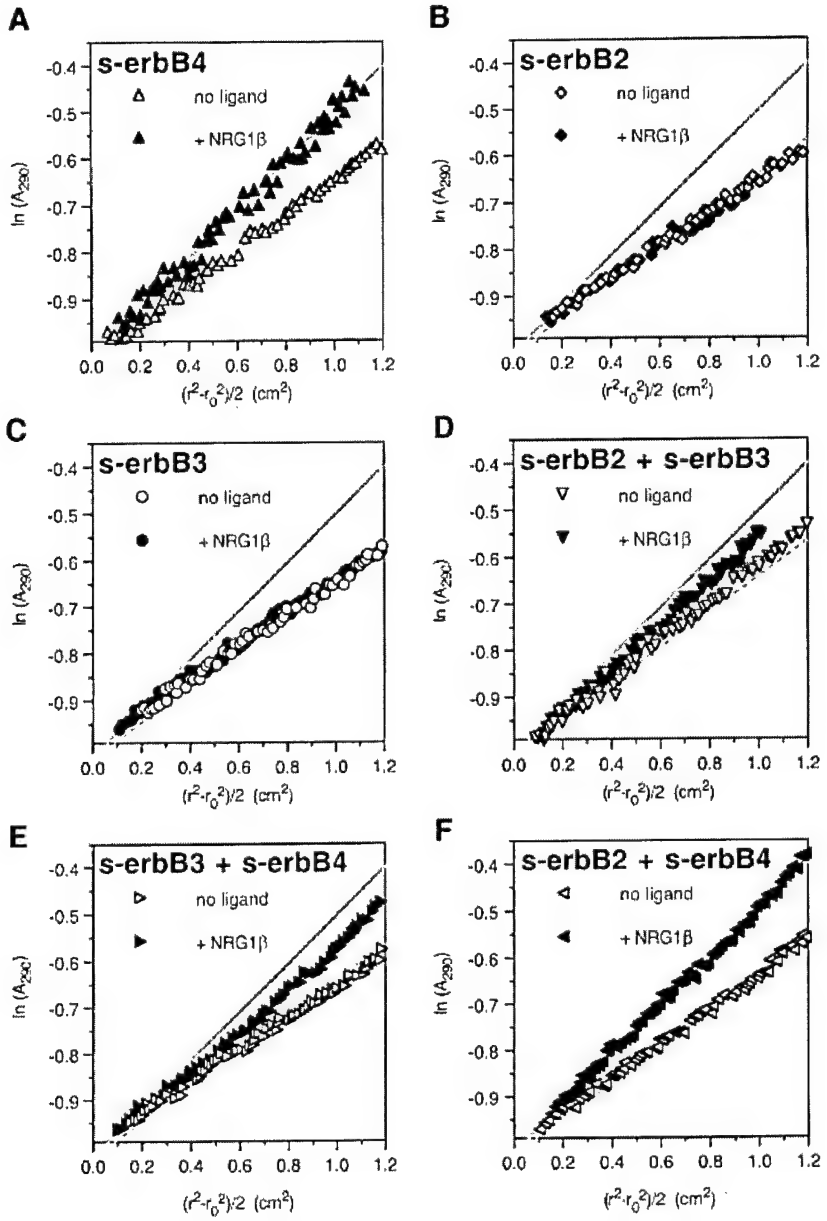

Fig. 7. Plots of ln(Abs) against $(r^2 - r_o^2)/2$ for different pairwise mixtures of s-erbB2, s-erbB3 and s-erbB4 with (open symbols) and without (filled symbols) added NRG1-β1. (**A**) The increase in gradient of the ln(Abs) against $(r^2 - r_o^2)/2$ plot that results from NRG1-β1-induced homo-oligomerization of s-erbB4. Lines corresponding to these data are superimposed in gray on each other graph in the figure. (**B** and **C**) NRG1-β1 fails to induce homo-oligomerization of s-erbB2 or s-erbB3. The data in (**D**) suggest that s-erbB2 and s-erbB3 may form very weak hetero-oligomers upon NRG1-β1 addition. As seen for s-erbB1 and s-erbB2 in Figure 5B, the data in (**E**) argue that s-erbB3 does not form hetero-oligomers with s-erbB4. The correspondence (**F**) of the line for the s-erbB2/s-erbB4 + NRG1-β1 sample with that for NRG1-β1-induced s-erbB4 oligomers shown in (A) indicates that NRG1-β1 can induce formation of s-erbB2–s-erbB4 hetero-oligomers (see text for details). Experiments were performed with a total s-erbB concentration of 10 μM, to which was added a 2-fold molar excess of NRG1-β1.

Brown *et al.*, 1994; Lemmon *et al.*, 1997). However, the data in Figure 3A suggest that the s-erbB1 used here dimerizes at least 15-fold more strongly upon EGF binding than material used in our earlier studies. Whereas the K_D for dimerization of a 1:1 EGF:s-erbB1 complex was estimated previously as 3.3 μM (Lemmon *et al.*, 1997), in which case it would be <50% dimeric in Figure 3A, the protein used in this study remained completely dimeric at concentrations as low as 250 nM (not shown). This difference may reflect the fact that, rather than using chaotropes to elute the protein from immunoaffinity columns, s-erbB1 produced for this study was purified under milder conditions, using metal affinity chromatography (see Materials and methods).

The K_D value reported for s-erbB3 binding to the EGF domain of NRG1-β1 (249 nM; Table I) is ~10-fold weaker than the value reported for its binding to full-length NRG1-β2 in analytical ultracentrifugation studies (Horan *et al.*, 1995). This difference may reflect the use of alternative NRG1-β isoforms in the two studies or, more likely, a contribution to s-erbB3 binding by regions of full-length NRG1-β2 outside the EGF domain (although the

Table II. Summary of ligand-induced s-erbB oligomers observed

	s-erbB1		s-erbB2		s-erbB3		s-erbB4	
	EGF	NRG1-β1	EGF	NRG1-β1	EGF	NRG1-β1	EGF	NRG1-β1
s-erbB1	homo	–	–	–	–	–	–	–
s-erbB2			–	–	–	hetero (weak)	–	hetero
s-erbB3					–	–	–	–
s-erbB4							–	homo

EGF domain is sufficient for all known biological activities of NRG1; Holmes *et al.*, 1992). In agreement with our findings (Figures 4C and 7), Horan *et al.* (1995) did not detect s-erbB3 homodimerization or s-erbB2–s-erbB3 heterodimerization upon NRG1-β2 binding.

Implications for erbB receptor oligomerization

As stated in the Introduction, we set out to test the hypothesis that the mechanism of erbB receptor transmodulation involves simple formation of receptor heterodimers upon binding to one or another bivalent ligand (Alroy and Yarden, 1997; Lemmon *et al.*, 1997; Tzahar *et al.*, 1997). We found that, in common with almost every other RTK extracellular domain that has been studied (Lemmon and Schlessinger, 1994; Heldin, 1995), the erbB1 and erbB4 extracellular domains form homo-oligomers upon binding to their respective ligands (EGF and NRG1-β1). As with other well characterized examples, this homo-oligomerization may be driven by bivalent erbB ligand binding. However, we could only detect the formation of one of the six possible pairwise s-erbB hetero-oligomers; s-erbB4 forming co-oligomers with s-erbB2 upon NRG1-β1 binding. EGF did not induce any s-erbB oligomer other than s-erbB1 homodimers, and our data suggest that the one hetero-oligomer that we could detect (s-erbB2–s-erbB4) is likely to be larger than a dimer.

These observations suggest that the simple erbB receptor heterodimerization hypothesis, in which ligand binding drives the heteromeric association of two different erbB receptors through their extracellular ligand-binding domains, is false. Instead, our findings argue that the mechanisms of ligand-induced erbB receptor homo- and hetero-oligomerization must be fundamentally different. In particular, the fact that ligand-induced erbB1 and erbB4 homo-oligomerization can be recapitulated with the isolated extracellular domains of these receptors, while hetero-oligomerization cannot, suggests that regions outside the extracellular domain are required for heteromeric, but not homomeric, interactions of the intact forms of these receptors.

A model for 'homodimer-nucleated' erbB receptor transmodulation

There are ~10^4–10^5 erbB1 or erbB4 receptors on the surface of a typical EGF- or NRG-responsive cell. For a cell with a radius of 8 μm, this receptor density translates to an effective local concentration of 0.1–3 μM at the very least. More reasonable estimates that account for orientation effects would be 10–100 times higher (Grasberger *et al.*, 1986). All experiments presented herein were performed with s-erbB proteins at concentrations of 4–10 μM, mimicking the effective erbB receptor concentration at the cell surface. Since liganded s-erbB1 and s-erbB4 homo-oligomerize so strongly under these conditions, we suggest that homo-oligomerization of the intact membrane-anchored receptors is likely to be the first response to ligand binding *in vivo*. It seems unlikely that ligand-induced hetero-oligomerization events that we cannot detect in the studies described here (driven by regions outside the extracellular domains) would compete with these strong, directly ligand-induced, homomeric interactions. We therefore suggest that the ligand-induced erbB receptor hetero-oligomers seen in many studies of intact erbB receptors are 'nucleated' by ligand-induced erbB1 or erbB4 homo-oligomers, and most probably represent something larger than a heterodimer. Huang *et al.* (1998) have suggested a similar model, as outlined in the Introduction, in which a ligand-induced homodimer of one receptor (e.g. erbB1) transactivates a second receptor (e.g. erbB2) by inducing its dimerization. In the resulting heterotetramer, the two molecules of the second (unliganded) receptor could activate one another through trans-autophosphorylation, and may be identical or different [if different, the 'secondary dimerization' observations made by Gamett *et al.* (1997) could be explained]. A 'homodimer-nucleated' hetero-tetramer model of this sort could explain the initially surprising finding that a kinase-negative mutant of erbB1 is nonetheless able to mediate EGF-induced transmodulation of erbB2 (Wright *et al.*, 1995). According to the model, an EGF-induced homodimer of the erbB1 mutant would transactivate erbB2 by inducing erbB2 homodimerization (and consequent activation) within the context of a heterotetramer—the kinase activity of erbB1 would not be required. The model could also explain how an erbB2 mutant with its intracellular domain deleted can inhibit transmodulation of endogenous erbB2 in a dominant-negative manner (Jones and Stern, 1999).

While this homodimer-nucleated heterotetramer model may explain transmodulation mediated by erbB1 or erbB2, it cannot readily explain the formation of erbB2–erbB3 hetero-oligomers. We and others (Horan *et al.*, 1995; Tzahar *et al.*, 1997) have failed to detect NRG-induced homodimerization of the erbB3 extracellular domain using biophysical or cross-linking methods. However, NRG-induced homo-oligomerization of intact (or truncated) erbB3 in cells has been detected in chemical cross-linking studies (Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1997). Unlike erbB1 or erbB4, erbB3 appears to require more

than just the extracellular domain for its ligand-induced homo-oligomerization. Tzahar *et al.* (1997) have presented evidence suggesting that transmembrane domain interactions may be important for both homo- and hetero-oligomeric interactions of erbB3. An NRG-induced erbB3 oligomer, stabilized by such interactions, could transmodulate erbB2 by inducing its 'proxy' dimerization in the model discussed above (see also Huang *et al.*, 1998).

Relationship of hetero-oligomer formation to ligand binding

Despite the fact that it does not bind either ligand independently, overexpression of erbB2 increases the NRG-binding affinity of cells that express erbB3 (Sliwkowski *et al.*, 1994; Karunagaran *et al.*, 1996) and the EGF-binding affinity of cells that express erbB1 (Karunagaran *et al.*, 1996). In an effort to understand these effects, Sliwkowski and colleagues investigated how forced heterodimerization of erbB receptor extracellular domains alters their ligand-binding properties. Hetero- (and homo-) dimerization was forced by fusing erbB receptor extracellular domains to the (dimeric) hinge and F_c portions of IgG_1 heavy chain. Heterodimeric IgG fusions containing the erbB2 extracellular domain alongside that of erbB3 or erbB4 bound NRG1-β significantly more strongly than erbB3 or erbB4 homodimer fusion proteins (Fitzpatrick *et al.*, 1998; J.T.Jones *et al.*, 1999). In contrast, a heterodimer containing the extracellular domains of erbB2 and erbB1 was indistinguishable from the equivalent erbB1 homodimer in its binding to EGF, TGF-α, HB-EGF or betacellulin (J.T.Jones *et al.*, 1999). This difference suggests that erbB2 enhances NRG and EGF binding through distinct mechanisms. While NRG binding may be enhanced simply by receptor extracellular domain heteromerization, some other mechanism must be invoked for the enhancement of cellular EGF binding by overexpression of erbB2 (Karunagaran *et al.*, 1996). Our studies of s-erbB oligomerization suggest a similar distinction: while the isolated extracellular domains cannot recapitulate ligand-induced erbB1–erbB2 hetero-oligomerization, at least NRG-induced erbB2–erbB4 heteromerization could be reproduced with the soluble s-erbB proteins studied here.

Conclusions

Regardless of the precise mechanism of ligand-induced erbB receptor hetero-oligomerization, the results presented here show that isolated extracellular domains reproduce ligand-induced homomeric interactions of erbB receptors more faithfully than their reported heteromeric interactions. This finding alone argues that the mechanisms for homo- and hetero-oligomerization of the erbB receptors must differ. Our data therefore provide strong evidence against the simple heterodimerization hypothesis that we set out to test. Rather, in agreement with suggestions made by other groups (Gamett *et al.*, 1997; Huang *et al.*, 1998), we suggest that the ligand-induced erbB homo-oligomers that can be formed with isolated extracellular domains nucleate larger erbB hetero-oligomers through interactions that may also involve other regions of the receptor. Transphosphorylation within these larger 'homodimer-nucleated' hetero-oligomers may be responsible for erbB receptor transmodulation.

Materials and methods

Generation of s-erbB constructs

A fragment of human erbB1 cDNA directing expression of residues 1–642 (1–618 of the mature sequence), followed by a hexahistidine tag and stop codon, was subcloned into pFastBac1 (Life Technologies Inc). The 1955 bp fragment was generated by PCR, introducing a unique *Bgl*II site immediately before the initiation codon and a unique *Xba*I site that follows the introduced stop codon. The 1955 bp *Bgl*II–*Xba*I-digested PCR product was ligated into *Bam*HI–*Xba*I-digested pFastBac I. To minimize the risk of PCR artifacts, a 1260 bp *Eco*RI–*Apa*I fragment of this PCR-derived clone was swapped for the equivalent region from the original erbB1 cDNA. A fragment of human erbB2 cDNA, directing expression of residues 1–647 (1–628 of the mature sequence), was generated similarly. In this case, a unique *Xba*I site was introduced before the initiation codon, and a unique *Hin*dIII site was introduced after the histidine tag and stop codon. The 1980 bp *Xba*I–*Hin*dIII-digested PCR product was ligated into *Xba*I–*Hin*dIII-digested pFastBac I. An 1880 bp internal fragment of this PCR product, extending from an *Nco*I site at the initiation codon to a unique *Sph*I site, was then swapped for the equivalent fragment from the original erbB2 cDNA.

Fragments encoding human erbB3 residues 1–639 (1–620 of the mature protein) and human erbB4 residues 1–649 (1–624 of the mature protein), with a unique *Bam*HI site at one end and an *Xba*I site at the other, were generated by PCR, and ligated into *Bam*HI–*Xba*I-digested pFastBac I. The sequence of all PCR-derived fragments and their cloning boundaries were confirmed by automated dideoxynucleotide sequencing methods.

Protein production

Typically, 5–10 l of Sf9 cells were grown as a suspension culture in Sf900-II medium (Gibco-BRL) using multiple 1 l spinner flasks that each contained <500 ml of medium (to ensure adequate aeration). At a cell density of 2.5×10^6 cells/ml (viability >98%), freshly amplified high-titer virus stock was added to a multiplicity of infection (m.o.i.) of ~5. Cultures were incubated at 27°C for a further 96 h. Clarified conditioned medium was concentrated 2-fold, and then diafiltered against 3.5 vols of 25 mM Tris–HCl, 150 mM NaCl, pH 8.0 (buffer A), using a Millipore Prep/Scale-TFF 30 kDa cartridge. The solution was concentrated further to ~300 ml prior to loading onto a 5 ml Ni-NTA Superflow column (Qiagen). After extensive washing with buffer A, the column was washed sequentially with two column volumes of buffer A containing 30, 50, 75, 100 and 300 mM imidazole, pH 8.0. Typically, most s-erbB protein eluted in the 75 and 100 mM fractions. Fractions were concentrated in a Centriprep 30 (Amicon), and loaded onto a Pharmacia Superose 6 gel filtration column in 25 mM HEPES pH 8.0, 100 mM NaCl, from which they eluted as ~85 kDa species. For s-erbB1 and s-erbB4, appropriate gel filtration fractions were pooled, diluted 1.5-fold with 50 mM MES pH 6.0, and were loaded on to an BioScale-S2 cation exchange column (Bio-Rad) pre-equilibrated with 25 mM MES pH 6.0. Protein was eluted with a gradient in NaCl, s-erbB1 eluting at ~200 mM NaCl and s-erbB4 at ~300 mM NaCl. Attempts to purify s-erbB2 and s-erbB3 by ion exchange led to precipitation of the proteins at the low salt concentration required for column binding. Purified s-erbB proteins were buffer exchanged into 25 mM HEPES, 100 mM NaCl, pH 8.0, concentrated to between 20 and 100 μM, and stored at 4°C. Purity was checked by SDS–PAGE (Figure 1), and concentrations were determined by absorbance at 280 nM using extinction coefficients calculated as described (Mach *et al.*, 1992) of 56 920/M/cm (s-erbB1), 62 460/M/cm (s-erbB2), 63 940/M/cm (s-erbB3) and 74 300/M/cm (s-erbB4). We previously had used quantitative amino acid analysis to measure a value of 58 500/M/cm for s-erbB1 from mammalian cells (Lemmon *et al.*, 1997); this value is within 3% of that calculated according to Mach *et al.* (1992). Calculated extinction coefficients of 18 780/M/cm (EGF) and 5920/M/cm (NRG1-β1) were also used for determination of ligand concentration.

Approximate final yields of purified protein from 1 l of conditioned medium were 1 (s-erbB1), 0.2 (s-erbB2), 1 (s-erbB3) and 0.5 mg (s-erbB4). Ligands used for this study were purchased from Intergen (human EGF) or R & D Systems (human NRG1-β1).

Multi-angle laser light-scattering (MALLS) studies

A DAWN DSP laser photometer from Wyatt Technologies (Santa Barbara, CA) was used for MALLS studies (Wyatt, 1993). The instrument was used in micro-batch mode, with samples being introduced into the flow cell via a 0.1 μm filter using a syringe pump. To avoid introduction of air bubbles, concentrated protein solutions were diluted to

working concentrations in degassed buffer, and samples were introduced into the flow cell via a low dead volume multi-port valve that was loaded with several samples and purged of air prior to a series of measurements. Scattering data at all 17 angles were collected until maximum stable scattering for a sample was seen, which can be achieved at flow rates of 2 ml/h with samples of ~300 µl. Scattering data were collected and analyzed using ASTRA software (Wyatt Technologies) supplied with the instrument. Relative weight-averaged molecular masses were determined from the scattering data collected for a given ligand:receptor mixture (once stabilized) using Debye plots, in which $R(\theta)/K^*c$ is plotted against $\sin^2(\theta/2)$, where θ is the scattering angle; $R(\theta)$ is the excess intensity (I) of scattered light at that angle; c is the concentration of the sample; and K^* is a constant equal to $4\pi^2n^2(dn/dc)^2/\lambda_0^4N_A$ (where n = solvent refractive index, dn/dc = refractive index increment of scattering sample, λ_0 = wavelength of scattered light and N_A = Avogadro's number). Extrapolation of a Debye plot to zero angle gives an estimate of the weight-averaged molecular mass ($\bar{M}_w$) (Wyatt, 1993). $\bar{M}_w$ is defined as:

$$\bar{M}_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i}$$

for n moles of i different species with molecular weight M_i.

In ligand titration experiments, the contribution of added ligand to the mass concentration was neglected (see also Lemmon *et al.*, 1997). Since we are interested in dimerization, i.e. only the 'fold increase' in $\bar{M}_w$, our results are not affected by the value of K^*, of which we are uncertain since we have not determined the extent of glycosylation of the s-erbB proteins accurately. MALLS data are therefore discussed in terms of 'fold increase' in $\bar{M}_w$ over that measured for s-erbB protein alone. Where estimates for $\bar{M}_w$ are reported, mass concentrations were converted from molar concentrations using the molecular weight suggested by the amino acid sequence, and assuming that s-erbB glycoproteins are 20% carbohydrate by mass.

Analytical ultracentrifugation studies

Sedimentation equilibrium experiments employed the XL-A analytical ultracentrifuge (Beckman). Samples were loaded into six-channel epon charcoal-filled centerpieces, using quartz windows. Experiments were performed at 20°C, detecting at 280–300 nm, using three different speeds (6000, 9000 and 12 000 r.p.m.), with very similar results. Solvent density was taken as 1.003 g/ml, and the partial specific volumes of the s-erbB proteins were approximated from their amino acid compositions and the assumption of ~20% carbohydrate as 0.71 ml/g for the purposes described here. Experiments were performed at 5–10 µM protein. Data were fit using the Optima XL-A data analysis software (Beckman/MicroCal) to models assuming a single non-ideal species for unliganded s-erbB proteins. When ligand was added, a two-species fit was used, in which one of the species was the excess ligand (partial specific volume 0.74 ml/g), which sediments as a 6 kDa (EGF) or 8 kDa (NRG) species (not shown). The molecular mass of the ligand species was fixed in these fits, while the mass and concentration of the receptor species were allowed to float. Goodness of fit was judged by the occurrence of randomly distributed residuals, examples of which are shown in Figure 4. For more complicated mixtures of receptors and ligands, simple qualitative interpretations of analytical ultracentrifugation experiments were made by inspection when possible (see Figures 5B, 6 and 7).

BIAcore studies

BIAcore binding experiments employed a BIAcore 2000 instrument, and were performed in 10 mM HEPES buffer, pH 7.4, that contained 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween-20 at 25°C. The hydrogel matrix of BIAcore CM5 Biosensor chips was activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-[3-(diethylamino)propyl] carbodiimide (EDC). EGF (at 200 µg/ml) in 10 mM sodium acetate, pH 4.0, or NRG1-β1 (at 200 µg/ml) in 10 mM sodium acetate, pH 4.8, was then flowed over the activated surface at 5 µl/min for 10 min. Non-cross-linked ligand was removed, and unreacted sites were blocked with 1 M ethanolamine, pH 8.5. The signal contributed by immobilized EGF or NRG1-β1 ranged from 150 to 400 RU, depending on the specific chip.

Purified s-erbB proteins at a series of concentrations were each flowed simultaneously over the EGF and NRG1-β1 (and mock/control) surfaces at 5 µl/min for 7 min, by which time binding had reached a plateau in each case. The RU value corresponding to this plateau was taken as a measure of s-erbB protein binding, and was corrected for background non-specific binding and bulk refractive index effects by subtraction of data obtained in parallel using the mock-coupled hydrogel surface. RU values were then converted into percentage maximal binding. This conversion was performed separately for each surface (since levels of immobilization varied); 100% binding was defined for an NRG surface as the highest corrected signal seen with s-erbB3 and s-erbB4 (which were always the same to within 10%), and for an EGF surface the highest corrected signal seen with s-erbB1. Buffer washes between runs were sufficient to bring the RU value back down to baseline. Data were plotted as s-erbB concentration against percentage maximal binding, and fit to a simple binding equation in ORIGIN (MicroCal) to estimate the K_D.

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